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<b>13. ABSTRACT (Maximum 200 Words)</b>  NKX3.1 is a prostate-specific homeobox gene that undergoes loss of heterozygosity in ~85% of prostate cancers, but one normal gene copy is retained in tumor cells. Haploinsufficiency of murine <i>Nkx3.1</i> is sufficient to permit prostatic hyperplasia and dysplasia later in life. Consistent with the findings in mouse models human NKX3.1 protein expression is substantially reduced in 80-90% of primary prostate cancer. Complete loss of NKX3.1 expression is seen mostly late in disease and in metastatic foci. Loss of NKX3.1 expression cannot be used as a prognostic marker in primary prostate cancer. Reduced expression of NKX3.1 in murine models and human prostate cancer occurs at the protein and not at the mRNA level. NKX3.1 promoter methylation is not a significant factor in reduced NKX3.1 expression in prostate cancer cell lines and tumors. In tumor model systems NKX3.1 has tumor suppressor properties and inhibits growth of human tumor cell xenografts in immunodeficient mice. NKX3.1 is therefore a candidate gatekeeper gene for human prostate cancer and an important focus of potential targeted therapies for prostate cancer.				
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## INTRODUCTION

This proposal focused on NKX3.1, a homeoprotein with prostate-specific expression in the adult. Since the human *NKX3.1* gene was described in 1997 several critical findings have underscored the potential importance of this gene in prostate cancer (1,2). Studies with mouse *Nkx3.1* deletion mutants have shown that NKX3.1 has growth suppression properties and is an important differentiation factor for prostatic epithelium (3). Mice with homozygous (-/-) deletion of *Nkx3.1* were viable and fertile. However, their prostate glands displayed epithelial hyperplasia early in life and dysplasia later in life along with late infertility. The protein components of prostatic secretions were altered in *Nkx3.1* (-/-) mice. An important finding of the gene deletion studies was that heterozygous (+/-) *Nkx3.1* mice also had a phenotype of prostatic epithelial hyperplasia and dedifferentiation. This suggested that if NKX3.1 expression was altered in human prostate cancer, decreased expression of the protein by disruption of one *NKX3.1* allele, promoter methylation, or other mechanisms that down regulated expression might be sufficient to influence prostate cancer phenotype.

Control of NKX3.1 expression in human tumors does not appear to resemble mechanisms seen with other genes that undergo promoter methylation in human tumors. Although the evidence has been thus far indirect, *NKX3.1* mRNA is not down regulated in human prostate cancer, but the protein expression is (4-6). Moreover, down regulation of NKX3.1 protein, but not mRNA was also observed in mice with compound deletions of *Nkx3.1* and *Pten*. Early prostate cancer lesions in these animal lost all expression of *Nkx3.1*, but retained mRNA expression (7).

Work from our lab during phase I of the project showed that NKX3.1 expression decreases during prostate cancer progression. Using an antiserum derived against purified recombinant NKX3.1 protein, we analyzed protein expression in benign prostatic hyperplasia, early stage prostate cancer, advanced local stage prostate cancer, locally recurrent hormone refractory disease, and metastatic disease. Whereas all normal prostatic epithelial cells express NKX3.1, there was a diminishing level of expression with more advanced disease. Approximately 30% of advanced and hormone-refractory prostate cancer had lost expression of NKX3.1 and nearly 80% of metastatic lesions were negative for immunohistochemical staining. The data suggest that loss of NKX3.1 expression may play a role in unrestricted growth of recurrent and metastatic disease. We therefore wanted to determine whether loss of NKX3.1 expression in localized prostate cancer had prognostic significance. Secondly, we also wanted to see if restoration of NKX3.1 expression to prostatic cells altered their phenotype by suppressing tumorigenesis.

During phase I of the project we have also characterized the expression of NKX3.1 in normal human tissues. In the mouse *NKX3.1* mRNA was found only in the three prostatic lobes and the bulbourethral gland (3). Human *NKX3.1* mRNA was seen in prostate and testis. We found NKX3.1 protein expressed in all normal prostatic epithelial cells, some testicular cells, and no other normal human tissues except rare pulmonary mucous glands and isolated patches of transitional epithelium in the ureter. NKX3.1 expression was restricted to a much greater degree than PSA expression (8). Therefore, the *NKX3.1* promoter may confer a higher degree of prostate-specific expression than the PSA promoter. PSA promoter is currently being used in the construction of prostate-specific gene therapy vectors(9-11). We wanted to isolate and characterize the promoter region of *NKX3.1*, both to study its role in the loss of NKX3.1 expression in prostate cancer and to identify the sequences responsible for prostate-specific expression for future construction of tissue-targeted vectors.



## **BODY**

### **(Organized according to the Statement of Work)**

**1. Determine if NKX3.1 expression is a clinical prognostic marker or a determinant of disease-free survival of patients undergoing radical prostatectomy for localized prostate cancer. (Years 1-2)**

#### **A. Identify tumor block regions for microarray cores.**

We identified a population from which we could access paraffin-embedded prostatectomy specimens and medical records for follow-up and outcomes data. The full study protocol, including access to the slides and blocks, was reviewed and approved by the Committee for the Protection of Human Subjects of Kaiser Permanente, Portland, OR. All patients who underwent radical prostatectomy for prostate cancer between 1970 and 1996 at KPNW were eligible for review of medical records and pathology slides. All patients were members of the Kaiser Health Plan and were treated at one of the two KPNW hospitals in Portland by members of the Northwest Permanente medical group. All surgical and pathological specimens were processed by members of the KPNW Department of Pathology. Less than 10% of KPNW patients with histologic diagnosis of prostate cancer during that time period chose to undergo therapy at other hospitals. Analysis was restricted to all patients for whom both records and slides were available for review; this material was missing from fewer than twenty cases. All medical records were re-abstracted for information specified prospectively in a database of clinically relevant information. Critical data elements entered into the database are listed in Table 1.

**Table 1**

#### **Data Collected by Medical Record and Pathology Review**

##### **Clinical Data – Diagnosis and Treatment**

- Race
- Age at diagnosis
- Method of diagnosis
- PSA at diagnosis (Y/N)
- TNM Stage including # of positive nodes
- Postoperative Radiation Therapy (Y/N)
- Postoperative Hormonal Therapy (Y/N)

##### **Follow-up Data**

- Progression (Local, Regional, Distant)
- Site of Progression
- How was Progression determined
- Systemic Therapy (Orchiectomy, DES, etc)

##### **Pathological Data**

- Gleason Score
- Prostatic Intraepithelial Neoplasia (Y/N)
- Perineural Invasion (Y/N)
- Urethral Margin Involved (Y/N)
- Seminal Vesicle Invasion (Y/N)

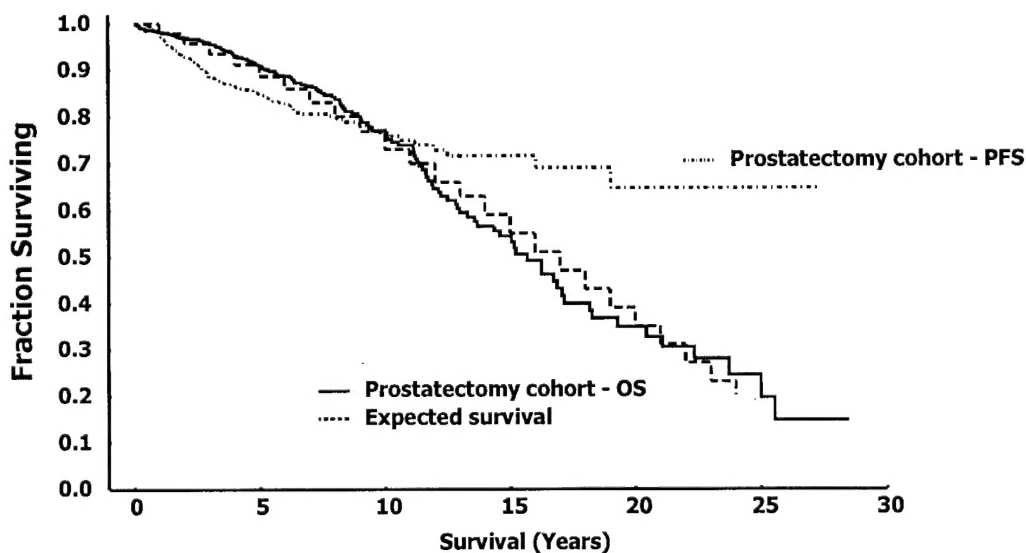
To provide a benchmark for comparison with the survival of patients in this series, we estimated the overall survival for a life table population of white males in Oregon (1989-91) using a distribution by age group the same as that in our cohort and obtained an expected median survival

time of 16.2 years. Life table data was obtained from the Centers for Disease Control (<http://www.cdc.gov/nchs/products/pubs/pubd/lftbls/decenn/1989-91.htm>).

Curves for survival and PFS from the date of diagnosis were calculated using the method of Kaplan and Meier (12) stratified by the factors in Table 2. Univariate analyses were performed to determine the patient characteristics related to PFS and survival by testing the statistical significance of the differences between curves using generalized Wilcoxon tests (13), since this test does not require an assumption of proportional hazards. Multivariate Cox regression models were fit to survival and PFS data from date of diagnosis separately to determine the combination of patient characteristics best related to outcomes. The models were fit in stepwise fashion beginning with all the statistically significant factors identified from the univariate analyses. A backward model selection method was applied in which non-significant covariates were eliminated step-by-step, so that the final Cox regression models include only statistically significant covariates. In the fitting of the Cox models, interrelationships among the covariates are taken into account, so that if one factor (e.g. Gleason score) was statistically significant, another factor might lose significance because it is correlated with Gleason score. In comparing the outcomes from this study with other studies, survival and PFS times have been measured from date of surgery to achieve comparable starting points. Post operation PFS and overall survival rates at 5, 7 and 10 years (including 95% confidence intervals) were calculated by using Kaplan-Meier product limit estimates and comparisons were made with published series of treatment for localized prostate cancer by Catalona (14), Gerber (15), Hull (16), and Barry (17).

The distribution of the demographic and clinical characteristics of the 750 patients in the study and percentiles of OS and PFS are shown in Table 2. The median age at diagnosis was 64 years and 53% of the patients were in the 60-69 year age group. All tumors were assessed histologically and staged according to the extent of organ involvement and presence of extracapsular extension. Approximately 80% of the patients had Stage T1 or T2 disease, with disease limited to the prostate at time of surgery. The median Gleason score was 6 and 27% (205) of patients had scores of 5 or less, while 40% (298) of patients had scores  $\geq 7$ . Other prognostic features found histologically

**Fig 1**  
**Survival of the Prostatectomy Cohort**



were perineural invasion in 42% (318) of patients and positive urethral margins in 6.4% (48) of patients. The men were predominantly Caucasian (96%), reflecting the population distribution of Portland, OR. During the follow-up period 18% (137) of patients developed progressive disease with either distant metastases (57%) or local recurrence (42%). A total of 20% (149) of patients died from all causes and only a small number, 18 patients (12% of deaths) could be counted as prostate cancer deaths. Figure 1 shows the survival and disease-specific survival of the cohort compared to a cohort of healthy men in Oregon of the median age of cohort.

Table 2 gives the quartiles of OS and PFS times according to groupings of patients by demographic and clinical characteristics. The median survival time for all patients was 15.7 years and 75% of patients had PFS times of 10.6 years or more. Age at diagnosis was significantly related to OS ( $P \leq 0.0001$ ), but not to PFS time ( $P = 0.53$ ). Survival was similar for patient groups up to 65 years, but declined thereafter; this was likely a consequence of counting deaths from all causes in the calculation of the survival curves. Using the male life table population of Oregon by age groups, the expected survival for a group of individuals with the same age distribution as in this study was 16.2 years, not very different from the median of 15.7 years for this cohort. T-stage and Gleason score were significantly related to both OS and PFS time. Patients with stage T1 or T2 disease have both a longer OS ( $P=0.001$ ) and PFS time ( $P < 0.0001$ ) than patients with T3 or T4 disease. Patients with Gleason scores  $\leq 6$  had significantly longer OS ( $P < 0.0001$ ) and PFS time ( $P < 0.001$ ) than patients with scores  $\geq 7$ . There were only 29 of non-Caucasian patients in the cohort and these had shorter OS and PFS times than Caucasian patients but the differences in time were not statistically significant ( $P = 0.48$ ,  $P = 0.13$ , respectively).

Of the 750 patients, 52% (388) had no evidence of invasive disease and these patients had a median OS of 16.3 years, which was not significantly longer ( $P = 0.24$ ) than the 15.7 year median time for patients with perineural invasion alone. However, there was nearly significant statistical evidence ( $P = 0.06$ ) that PFS times were longer for patients with no evidence of invasion. Patients with two or more types of invasion (perineural or urethral margin positive plus other) had significantly shorter OS and PFS times than patients with no invasion. Patients with seminal vesicle invasion had shorter OS and PFS times than patients with no invasion, which was statistically significant for PFS ( $P < 0.0001$ ), but not OS ( $P = 0.25$ ). There were only 18 patients with evidence of lymph node involvement ( $N_1$ ), but these patients had significantly shorter PFS times ( $P < .0001$ ) than patients with no involvement ( $N_0$ ).

Cox multivariate regression models were fit to investigate the relationship of multiple patient characteristics to outcomes, including only those individual patient characteristics demonstrating a statistically significant relationship to outcome ( $P \leq 0.05$ ) from the univariate analyses. The final models for survival and PFS include only statistically significant patient characteristics related to outcome ( $P < 0.05$ ).

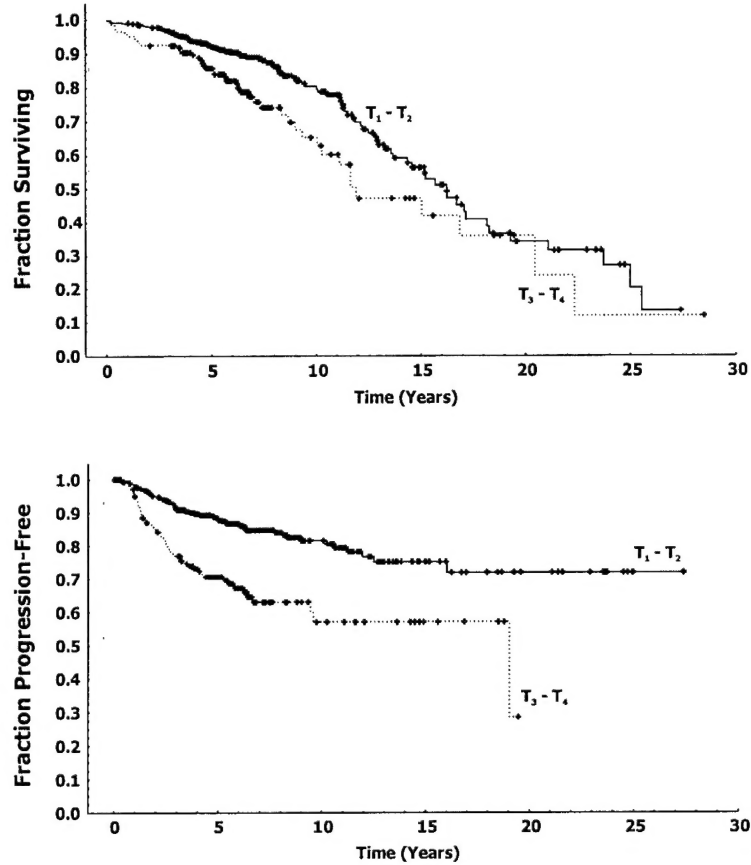
Table 3 gives the patient characteristics in the final Cox regression models for OS and PFS. For both OS and PFS, both Gleason score and T stage are statistically significant ( $P < 0.05$ ). The other statistically significant characteristic included in the final model for OS is age at diagnosis. From the regression coefficients and the code for each patient's characteristic (Table 3), the patient characteristics with an important favorable relationship to survival are: Gleason score  $\leq 6$ , tumor stage of T<sub>1</sub> or T<sub>2</sub>, and younger age at diagnosis. The former two parameters also had a favorable relationship to PFS. Figures 2 and 3 show the relationship effects of stage and grade to disease-free and overall survival.

**Table2**

**Relationship between population and clinical characteristics to overall survival (OS) and progression-free survival (PFS).**

Progression-free survival (PFS).										
Characteristic	No. pat (%).	No. death	Percentiles of OS			P value	No. progression	Percentiles of PFS		
			75	50	25			75	50	P value
<b>All Patients</b>	750 (100)	149	10.3	15.7	23.7	-	137	10.6	-	-
<b>Age at Diagnosis</b>										
45-	190 (25)	26	12.2	21.1	-		39	10.6	-	
60-	195 (26)	24	15.1	22.4	25.0		40	8.3	-	
65-	205 (27)	43	8.4	15.2	20.5	<0.0001	37	10.2	-	0.53
70 and over	160 (21)	56	7.4	11.4	13.6		21	6.3+	-	
<b>T stage</b>										
T1-T2	603 (80)	105	11.2	16.3	25.0	0.001	88	16.0	-	<0.0001
T3-T4	147 (20)	44	7.4	11.9	20.5		49	3.4	19.0	
<b>Gleason Score</b>										
≤6	450 (60)	71	12.2	18.2	25.5	<0.0001	53	19.0	-	<0.0001
≥7	298 (40)	76	8.3	11.9	17.1		83	4.9	-	
<b>Invasion status</b>										
No invasion	388 (52)	66	11.1	16.3	-		50	16.0	-	
Perineural Invasion alone	271 (36)	58	10.1	15.7	23.7	0.24	51	8.3	-	0.06
Perineural plus any other invasion	47 (6)	14	6.3	14.6	-	0.004	22	2.3	6.4	<0.0001
Urethral Margin Positive	48 (6)	15	7.1	14.6	17.1	0.03	17	2.9	11.2	0.0001
Seminal Vesicle Invasion	47 (6)	14	7.1	11.7	15.1	0.25	22	2.6	9.6	<0.0001
<b>Nodal status</b>										
N <sub>0</sub>	708 (98)	130	11.2	16.3	23.7	0.26	121	11.2	-	<0.0001
N1	18 (2)	5	8.7	11.7	-		8	2.7	-	
<b>Diagnosis method</b>										
TRUS	547 (73)	37	10.1+	-	-	<0.0001	85	9.6	-	0.47
Others	203 (27)	112	7.3	13.6	22.4		52	9.5	-	
<b>PSA at Diagnosis</b>										
Yes	526 (70)	38	10.1	-	-	<0.0001	80	6.4+	-	0.49
No	224 (30)	111	8.0	14.4	23.7		57	9.5	-	
<b>Post radiation therapy</b>										
Yes	71 (9)	29	6.4	12.2	-	0.01	18	9.6	-	0.35
No	679 (91)	120	11.1	16.3	23.7		119	11.2	-	
<b>Post hormone therapy</b>										
Yes	166 (22)	29	8.9	10.1	18.2	0.37	35	9.5	12.5	0.047
No	584 (78)	120	11.2	15.7	25.0		102	11.9	-	
<b>Race</b>										
Caucasian	721 (96)	144	10.5	15.7	23.7	0.48	130	11.2	-	0.13
Black, Asian	29 (4)	5	8.0	-	-		2	5.0	-	

**Fig 2**  
Effect of Stage on Progression-free and Overall Survival



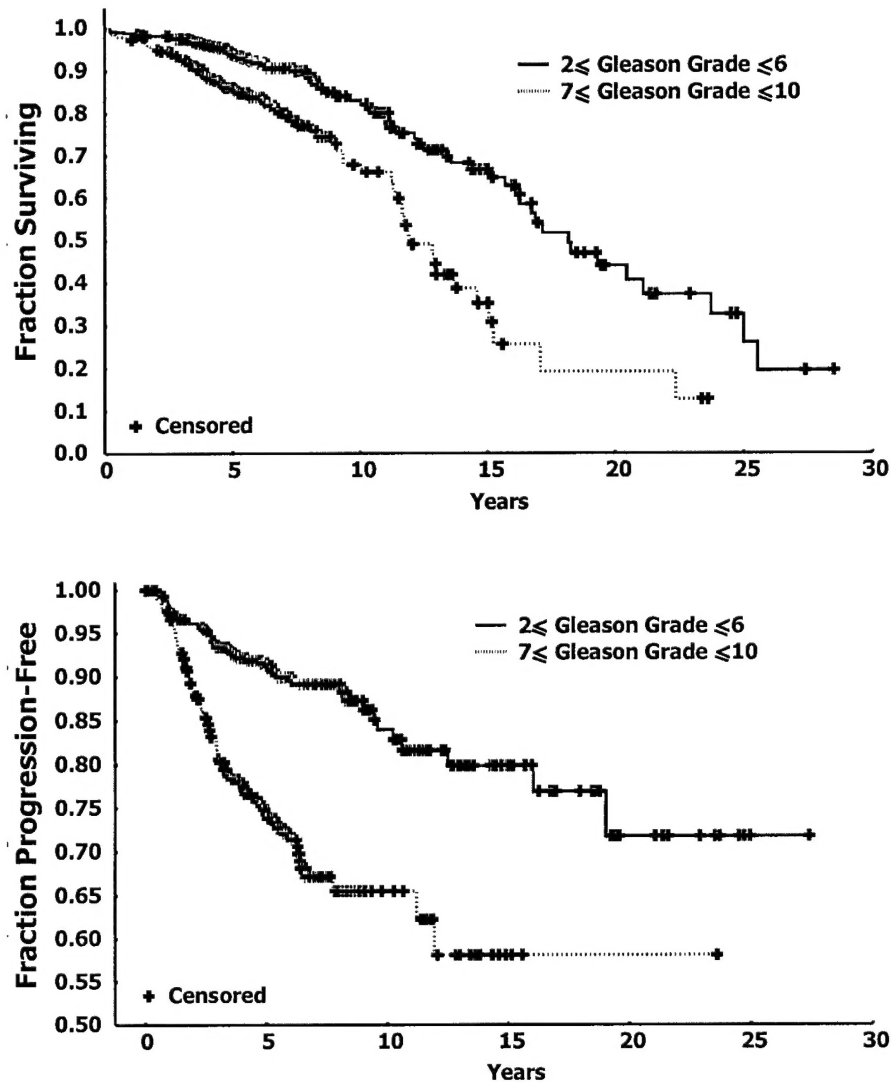
**Table 3**

**Results of the Cox Multivariate Regression Models**

Outcome	Characteristics	Code for analysis	Regression coefficient	P	Hazard ratio
OS	Gleason score	0= score $\leq$ 6 * 1= score $\geq$ 7	0.7319	<0.0001	2.08
	Age at diagnosis	1=45-64 * 2=65-69 3=70 and over	0.6374	<0.0001	1.89
	T stage	0=T1-T2 * 1=T3-T4	0.3619	0.0485	1.44
PFS	Gleason score	0= score $\leq$ 6 * 1= score $\geq$ 7	0.9360	<0.0001	2.55
	T stage	0=T1-T2 * 1=T3-T4	0.7243	0.0004	2.06

\*: Favorable

**Fig 3**  
**Effect of Gleason Grade on Progression-Free and Overall Survival**



## B. Prepare microarray.

A total of 551 patients from the 750 in the above-described cohort (all members of the Kaiser Foundation Health Plan) with a median age of 63.6 (range 45-92) years, who were treated for clinically localized prostate cancer by radical prostatectomy or transurethral resection (TURP) at one of two Kaiser hospitals in Portland, OR, between 1971 and 1996, were retrospectively evaluated and used for the TMA. Complete follow-up data were available from all patients including progression (median 5.3 years, range 0.5-20 years), overall (median 5.9 years, range 0.5-20 years) and tumor-specific survival (median 5.9 years, range 0.5-20 years). All patient identifiers were removed and replaced by unique study numbers, linked to the original identifiers by a single file kept under high security. Medical records for the entire cohort were abstracted at one time, 1999-2001, to assure uniform criteria for diagnosis, progression and staging. Before 1992 (pre-PSA era), progression was defined clinically based on the results of bone scans, chest x-rays and/or digital rectal examination. After 1992, progression was defined by increasing PSA serum concentrations in serial determinations following a postoperative PSA nadir value. Progression occurred in 111 (20.1%) of 551 the patients. Progression was defined by increasing PSA serum concentrations in 81 patients

(73%), by positive bone scans in 13 patients (11.7%), by lung metastases in 6 patients (5.4%) and by an increasing size of the palpable tumor in 9 patients (8.1%).

The specimens were from 498 patients treated by radical prostatectomy with a mean age of  $63.74 \pm 7.7$  years). They also included 53 transurethral resections (TURP's) from patients who were not eligible for radical prostatectomy mostly based on advanced age (mean age of  $73.41 \pm 8.9$  years). The pathologic stage of the radical prostatectomy specimens was obtained from the Kaiser Permanente Cancer Registry, which had used clinical records, operative and pathology reports. The slide with the least differentiated tumor area was selected for the tumor microarray (TMA). Because of their small size, Gleason grade rather than Gleason score was assigned to the specimens on the TMA sections [17]. The stage distribution of the radical prostatectomy specimens was pT2 (396 patients), pT3 (86 patients), and pT4 (16 patients) according to the criteria of the International Union against Cancer [18] and the American Joint Commission on Cancer. Lymph node metastases were present in 14 of the 428 patients in whom pelvic lymph node dissection was performed before radical prostatectomy. Neoadjuvant hormonal treatment (typically leuprolide given monthly for 1-3 doses) was given to 101 of 498 patients who underwent radical prostatectomy (mean duration  $49.7 \pm 19.7$  days, range 8-134 days). The TURP specimens were from tumors with clinical stage T1 (n=34), T2 (n=7), T3 (n=2), T4 (n=6), and TX (n=4). Patients with known distant metastases before TURP were not considered in this study. Further treatment after TURP included radiation therapy (n=18) and/or hormonal ablation (n=13). None of the patients in this group received any chemotherapy. Nonmalignant control tissues consisted of 86 TURP specimens from patients with benign prostatic hyperplasia (BPH).

The KPNW Cancer Registry recorded the pathologic staging employed in the 4<sup>th</sup> Edition of the *UICC Manual for Cancer Staging*. The prostate tissue microarray was constructed as previously described [5]. Briefly, one core tissue-biopsy (diameter 0.6mm) was taken from the least differentiated region of individual paraffin-embedded prostate tumors (donor blocks) and precisely arrayed into a new recipient paraffin block (35mm x 20mm) with a custom-built precision instrument (Beecher Instruments, Silver spring, MD). The core-tissue-biopsies were put into one of the two recipient blocks that defined one replicate TMA (Figure 1). Six replicate TMA arrays containing the identical set of tumors were constructed. After the block construction was completed, 5 $\mu$ m sections were cut with a microtome using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, New Jersey) to support the adhesion of the array elements. The presence of tumor tissue on the arrayed samples was verified on a hematoxylin-eosin-stained section. Originally, 732 donor tissue blocks were available for the construction of this TMA. Specimens from 181 tumors could not be included in the study because of incomplete follow-up data, lack of tumor in the arrayed sample (sampling error), damaged tissue (heat or crush artifacts), or a total lack of tissue at some array positions ('empty spots'). Therefore, all data in this study are based upon the analysis of 551 prostate cancer tissue specimens. The number of patients varies slightly between the individual marker analyses, because of variability in the number of interpretable specimens on consecutive TMA sections.

### **C. Stain and analyze microarray**

Staining was done according to our published procedures (6). For reasons explained by the data in section 2F we do not have a final result on the relationship between NKX3.1 expression and prostate cancer prognosis. This is due to the fact that NKX3.1 expression varies quantitatively, not qualitatively. Therefore we had to develop techniques to analyze quantitative differences in expression. These will then be applied to the microarrays to complete the analysis.



## **D. Section, stain and analyze 10% validation sample set from tissues that were incorporated in microarray**

This element was not performed – see 2F.

## **E. Section and stain biopsies from patients in radiation therapy cohort**

Recutting and staining of the biopsies provided very low yield of prostate tissue and was abandoned after a pilot trial in which we sectioned several archival biopsy blocks.

## **F. Statistical analysis of NKX3.1 expression data from the three cohorts**

### **2. Determine the mechanism of NKX3.1 down regulation in prostate cancer. (Years 1-2)**

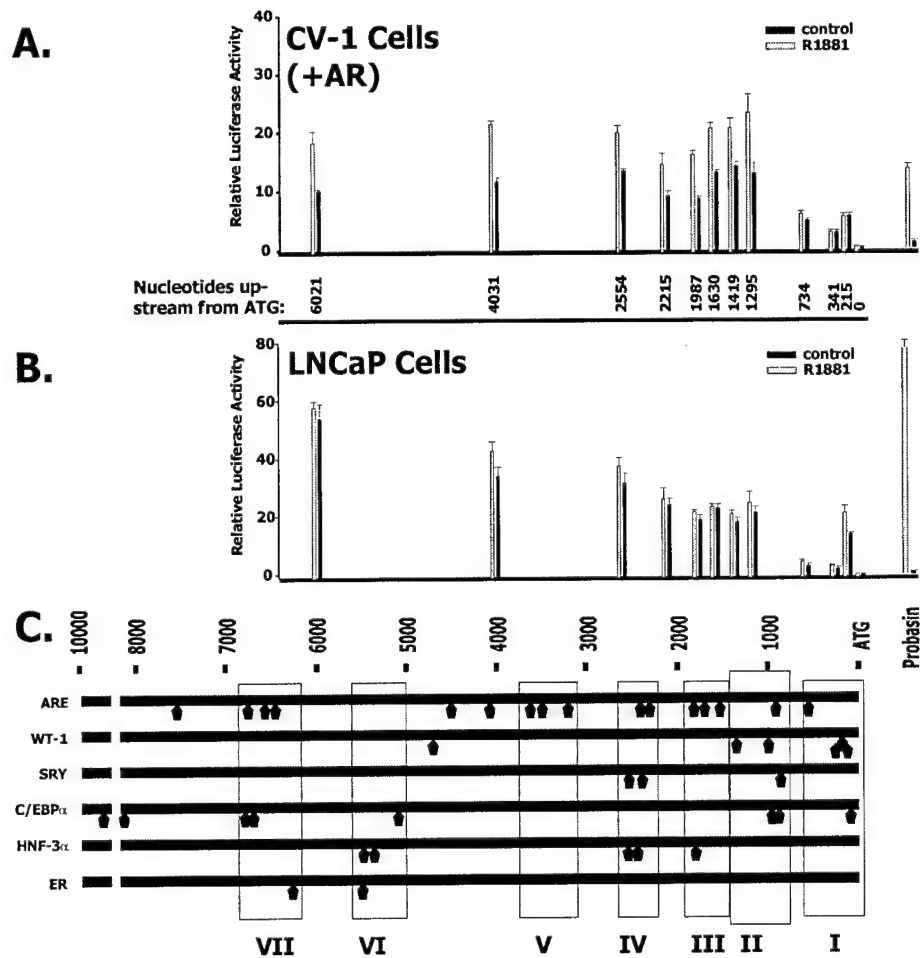
#### **A. Clone and sequence the 5' upstream region of *NKX3.1*.**

We have conducted preliminary analysis of the 5' upstream region of the *NKX3.1* gene and analyzed the androgen responsiveness and cell-type specificity of transcription conferred by different regions of the promoter. The sequences from the ATG to 10 kb upstream are in clone AC012574. The sequences extending from 5 kb upstream to 26 kb upstream are in clone AC022587. We have obtained BAC clones with these DNA fragments for use in constructing *NKX3.1* promoter reporter plasmids. Figure 4 shows preliminary analysis of *NKX3.1* promoter activity. All assays were controlled with *Renilla* and performed in triplicate in either CV-1 cells (Figure 4A) or LNCaP cells (Figure 4B) grown in charcoal-stripped serum with or without the synthetic androgen R1881 (1nM). In CV-1 cells cotransfected with an AR expression plasmid we observed uniform promoter activity among several *NKX3.1* promoter constructs whose length is indicated by the numbers between panels A and B in Figure 4. Note that all three panels in Figure 4 are drawn to scale and are aligned so that DNA binding regions of potential interest are aligned with the results of reporter gene assays. In LNCaP cells, where to date we have tested a broader range of constructs than in CV-1 cells, we detected a region between -215 and -734 that inhibited transcription. The -215/-734 region contains multiple WT-1 binding sites that may play an important role in *NKX3.1* expression (see below). Beyond -734 transcriptional activity gradually increased, suggesting that several regions of the promoter contribute to cell type-specific expression of *NKX3.1*. We also found quite striking the absence of androgen-stimulation of transcription in LNCaP cells by constructs extending to -6021. This strongly suggests that a major transcriptional inhibitory region lies upstream from -6021. We expect that this inhibitory region will repress transcription in the absence of androgen, but not in the presence of androgen.

Using data from the Human Genome Sequence and sequence provided by Cathy Vocke, Urology Branch, NIH who has sequenced the entire region of 8p21 that is deleted in prostate cancer, we have constructed a map of the promoter region of *NKX3.1*, concentrating to date on the proximal 10 kb. The entire 5' untranslated region upstream from the *NKX3.1* ATG extends for 19,600 nucleotides to the 3' end of *NKX2.6* an analogue of *NKX2.5* important for development of the pharyngeal pouch (18-20). There are several striking features about the 5' upstream sequence (Figure 4C). Although the analysis of the promoter regions and focus of our experiments will be driven by functional results of deletion studies, we have highlighted a number of transcription factor binding sites because of their potential importance in organ-specific expression or because of the frequency and location of their DNA-binding elements. These transcription factor-binding sites may draw our attention during the analysis. We provide a brief description of these transcription factors below as part of our annotation of the *NKX3.1* promoter region.



**Fig 4**  
**Analysis of NKX3.1 Promoter Activity**



#### i. Androgen Response Elements

Within the proximal 10 kb we found 16 candidate androgen-response elements (ARE). These elements are grouped nonrandomly and form at least four clusters. Clustering of AREs and occurrence of multiple copies suggest that, like other androgen-responsive genes, the binding of multiple AR molecules is required for maximal transcriptional activation. The ARE is comprised of two palindromic hexanucleotide half sites separated by a three-nucleotide spacer (21). There is substantial variation between the AREs seen in different promoter regions. However, single nucleotide differences in AREs affect the affinity of different receptors for DNA and can influence the relative effects of different steroid hormone receptors on specific promoter regions (22). In addition, the presence of multiple AREs in a promoter region causes tandem promoter binding and enhancement of AR specificity and action (23-25). In fact, individual AREs in general have low activity in in vitro assays of transcription. Androgen-induced transcriptional activity that mimics transcriptional activation seen in cells requires the presence of a number of AREs arranged in the correct spatial orientation (26-28). Therefore, specificity for hormone action must be dictated to some extent by other regions of the receptor and interaction with other cis acting elements that

participate in transcriptional initiation (29,30). Moreover, there are two classes of ARE that cooperate to enhance AR binding to DNA and may be critical for determining specificity of hormonal responsiveness of a promoter. AR will bind to guanine residues indicated by underlined letters in the sequence RGAACA-NGN-TGTNCT. ARE will also bind to a second class of ARE, RGGACA-NNA-AGCCAA, that mediates cooperation between adjacent bound receptors and enhances the specificity of androgen responsiveness (31).

**Table 4**  
**ARE Sequences in NKX3.1 Promoter**

ARE - I: R G A A C A N G N T G T N C T															ARE - II: R G G A C A N N A A G C C A A															
Position																														
-712	G	T	A	T	G	C	T	G	A	T	G	T	T	C	T															
-940	C	A	T	C	A	T	C	T	C	T	G	T	T	C	C															
-1548																A	G	C	C	A	G	C	T	T	T	G	C	C	A	A
-1609	A	T	T	A	A	A	G	A	A	T	G	T	T	C	T															
-1710	A	A	A	A	G	A	T	G	T	A	A	C	A	G	G															
-2354	A	A	A	A	T	C	T	T	T	T	G	T	T	C	T															
-2418	T	A	A	A	C	A	A	A	G	A	G	T	T	C	T															
-3171	A	C	A	A	G	A	A	T	A	T	G	T	T	C	T															
-3540																C	T	G	T	C	C	C	T	C	T	C	C	C	A	A
-3563	A	A	T	A	C	A	C	C	C	T	G	T	T	C	C															
-4063	A	G	G	G	G	G	C	A	T	T	G	T	T	C	T															
-4438	T	C	A	G	G	G	T	T	G	T	G	T	T	C	C															
-6454	A	C	A	G	G	T	G	T	C	A	G	T	C	T	T															
-6608	A	G	G	A	C	A	A	C	C	T	G	G	G	T	T															
-6721	A	G	A	C	T	G	A	T	C	T	G	T	T	C	T															
-7394	G	T	A	T	C	G	C	C	A	T	G	T	T	G	C															

Putative AREs in the 10kb 5' upstream sequence of *NKX3.1* are shown in Table 4. Letters in bold in each putative ARE match the consensus sequence which is quoted according to Reid et al (31). Each ARE has a two semipalindromic hexanucleotides that flank a 3-nucleotide spacer that is shaded.

We also found two potential estrogen response elements at -5312 and -6270. The effect of estrogen on *NKX3.1* expression is unknown. However, expression of *NKX3.1* was found in bronchial mucous glands in both males and females and in ureter (6). It may be that to sustain expression of *NKX3.1* in these organs in females, estrogen response is required. It was also interesting that we found activation of *NKX3.1* expression in some human breast cancers. The estrogen-dependence of this expression remains to be determined.

## ii. WT1 (-236, -254, -293, -1000, -1306, -4769)

WT1 tumor suppressor gene encodes a zinc finger transcription factor that is inactivated in the germline of children with genetic predisposition to Wilm's tumor and in a subset of sporadic cancers (32). WT1 is expressed at an early stage of genital ridge formation, and knockout mice lacking functional copies of WT1 fail to develop gonads regardless of their sex. WT1 is expressed in DU-145 and PC-3 cells, two cell lines from advanced metastatic prostate cancer. It is not expressed in LNCaP and MDAPCa2b, two androgen-sensitive metastatic prostate cancer cell lines (33). Alternative pre-mRNA splicing at two sites in the WT1 gene produces four protein isoforms. The first alternative splice site inserts or omits 17 amino acids N-terminal to the zinc finger domain. The second alternative splice site determines the presence of a highly conserved Lys-The-Ser (+/- KTS) sequence in the linker between zinc-fingers 3 and 4. These alternative +/-KTS splicing WT1

products have very different developmental functions as has been shown by selective gene targeting studies (34). WT1 is known to regulate several genes involved in sex determination and differentiation such as SRY, Mullerian-inhibiting substance, amphiregulin, and androgen receptor (35-37).

Three different WT1 DNA-binding consensus sequences have been described. The Zn-finger domain of WT1 (+KTS) binds with higher affinity than WT1 (-KTS) to a GC-rich EGR1-like DNA-binding element (38). The GC-rich EGR1-like sequence regulates the E-cadherin promoter, among other genes, an association that may be relevant in prostate cancer (39). A second potential DNA-binding sequence is comprised of TCC repeats (40). Expression of the androgen receptor gene is inhibited by binding of WT1 to either GC-rich EGR1-like sequences or TCC repeats located in the promoter region of the *AR* gene (36). The WT1 (-KTS) zinc finger domain has high affinity for the sequence 5'-GCGTGGGAGT-3' (41). This binding site, termed WTE, is found in promoters of *BCL-2*, amphiregulin, and *SRY* (35,37,42). Because WT1 influences sex determination and is a tumor suppressor, we will determine the role of the WT-1 binding sites present upstream from *NKX3.1* (see(43) for recent review). In particular we will focus on the cluster of three WT1 sites within 300 bp of the ATG).

Of great interest is the fact that the early growth response gene product, EGR-1, is a transcription factor that binds to DNA sequences similar to those that bind WT-1 (44,45). *Egr-1* gene deletion in mice results in suppression of prostate tumorigenesis caused by the SV40 T-antigen transgene of the TRAMP mouse (46). It is intriguing to speculate that if EGR-1 is inactivated in prostate cancer it may contribute to down regulation of *NKX3.1* expression by failing to bind to the WT-1 sequence or by binding of an inactive protein that has a dominant negative effect.

### iii. Hepatocyte Nuclear Factor (HNF)-3 $\alpha$ (-1770, -2255, -2285, -5225, -5236)

We have identified two clusters of HNF-3 $\alpha$  binding sites around -2200 and -5200 bp upstream from the ATG of *NKX3.1*. HNF-3 $\alpha$  belongs to the winged helix/forkhead family that includes HNF-3 $\beta$ , HNF-3 $\gamma$ , BF-1, and BF-2. HNF-3 proteins were originally identified as factors mediating hepatocyte-specific transcription of the transthyretin and  $\alpha$ 1-antitrypsin genes (47-50). The HNF-3 proteins have a strong homology with *Drosophila* forkhead proteins in their conserved 110-amino acid winged helix DNA binding domain. The DNA binding domain VAWTRTTDRYTY directs monomeric recognition of a DNA consensus binding sequence (51). The promoter regions of numerous liver-specific genes have been shown to contain this consensus sequence (52).

The HNF-3  $\alpha$ ,  $\beta$  and  $\gamma$  proteins are sequentially expressed during development of the endoderm as well as in cells of the notochord and ventral neural epithelium (47,53). In adult, HNF-3 $\alpha$  is known to be expressed in liver, intestine, stomach, pancreas, lung, prostate, seminal vesicle, and bladder (47,54). Importantly, HNF-3 $\alpha$  expression may be hormone-dependent since its expression is drastically reduced in the rat prostate after castration (54). HNF-3 family members may also be associated with the action of steroid hormones. HNF-3 proteins are accessory factors required for transcription of the phosphoenolpyruvate carboxykinase gene by glucocorticoids (55). HNF-3 proteins also synergize with estrogen receptor to induce activation of the vitellogenin B1 promoter (56). We note that in the *NKX3.1* promoter two HNF-3 $\alpha$  sites map quite near an ARE cluster and WT1 sites (see region IV in Figure 2C).

### iv. SRY (-857, -2267, -2434,)

Sex determination in the male is triggered by the *SRY* gene located at interval 1A1 of the Y chromosome. SRY protein controls the expression of several genes and initiates a cascade of events

leading to testicular differentiation and male development (57). SRY is expressed primarily in testis, but also in prostate (58,59). SRY is also expressed in LNCaP prostate cancer cells where its expression is suppressed by R1881, but SRY is not expressed in DU-145 or PC-3 prostate cancer cells (58,59). SRY expression was lost in 38% of prostate cancer tissues (60).

SRY contains a high-mobility group (HMG) box that is shared by several transcription factors such as SRY-related HMG box (SOX) proteins that also play an important role in determining the structure and function of sex organs (43). The DNA-binding consensus sequence of SRY is (A/T)AACAA(T/A) (61). The core DNA-binding consensus sequence of SOX is the same as that of SRY (62). Subtle differences in the sequence of DNA regions flanking the core DNA-binding sequence determine affinity differences of SOX and SRY for their respective binding sites. SRY recognizes conserved DNA binding sites in the promoters of sex-specific genes such as P450 aromatase and Mullerian inhibiting substance (63). It has been proposed that SRY affects on transcriptional activity by modifying the conformation of DNA strands (64,65). We have found three potential SRY-binding sites in the *NKX3.1* promoter. The importance of these sites for transcription in cultured cells will be examined as described below.

#### **v. CCAAT/Enhancer Binding Protein (C/EBP) (-172, -935, -965, -5133, -6737, -6863, -8833, -9341)**

C/EBP family includes the transcription factors C/EBP $\alpha$  (C/EBP), C/EBP $\beta$  (NF-IL6), C/EBP $\gamma$  (Ig/EBP-1), C/EBP $\delta$  (NF-IL6b), C/EBP $\epsilon$ , and C/EBP $\xi$  (CHOP/Gadd153). C/EBP $\alpha$ ,  $\beta$ , and  $\delta$  are expressed primarily in liver, lung, and adipose tissues. C/EBP $\epsilon$  is expressed in myelocytes. C/EBP $\gamma$  and  $\xi$  are expressed ubiquitously. The expression of C/EBP $\beta$  is induced by inflammation or infection markedly in many tissues. Gene deletion experiments have shown that C/EBPs have diverse functions in many tissues (66,67). In particular, C/EBP expression is important in inflammation and immunity (68). C/EBP proteins have a highly conserved C-terminal basic leucine zipper (bZip) DNA-binding domain. The bZip domain is constituted from a basic region involved in DNA binding site recognition and an adjacent leucine zipper that mediates protein dimerization during DNA binding (69-71). The consensus binding site of C/EBP is the palindromic sequence ATTGCGCAAT, although naturally occurring C/EBP binding sites contain a well conserved half-site and a more divergent one. There are many evidences supporting that C/EBP proteins have an important role in the physiological functions of glucocorticoid (72-75). Interaction between C/EBP and androgen-driven transcription has not been demonstrated directly. A single report demonstrated the repressor effect of C/EBP on androgen activation of the dehydroepiandrosterone sulfotransferase gene promoter (76). Note that C/EBP $\alpha$  binding sites cluster near -1000 in a region that negatively regulates the *NKX3.1* promoter. Moreover, there are two C/EBP $\alpha$  binding sites upstream from -6021. These are candidate sites for down regulation of transcription in the absence of androgen, possibly to explain the lack of differential activity of the -6021 construct in the presence and absence of androgen.

#### **B. Perform primer extension to determine 5' end of mRNA.**

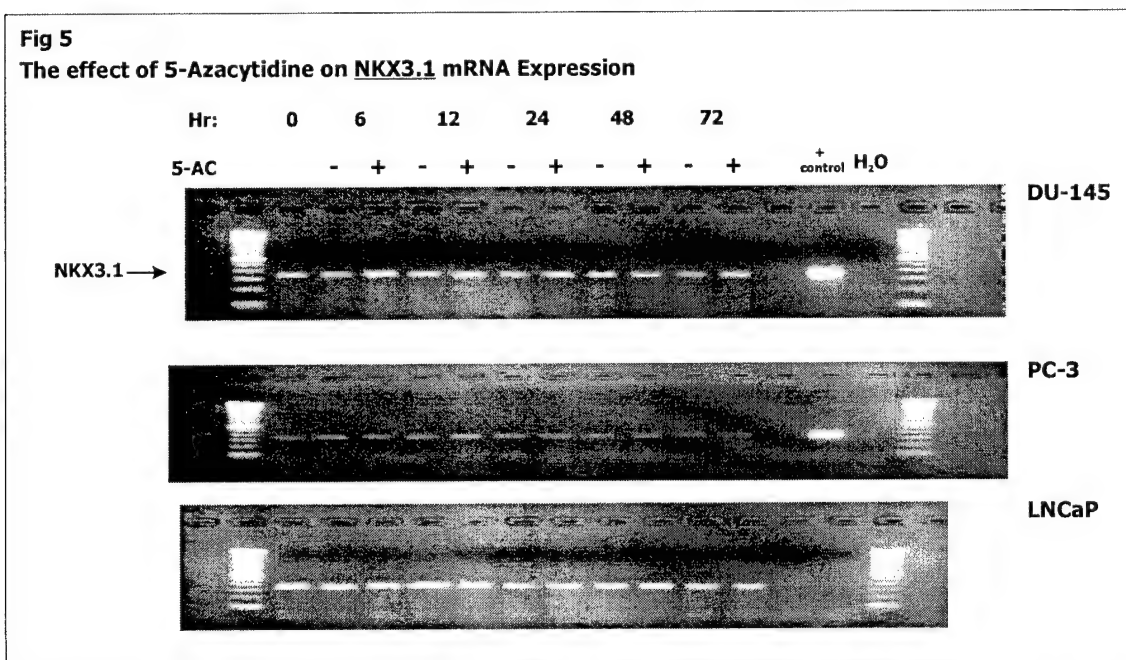
Due to results of experiments in the remainder of this element of the Statement of Work, this task was not pursued.

#### **C. Perform HpaII/MspI analysis of 5' upstream, exon I and intron I to identify areas that are differentially methylated in NKX3.1 + and - prostate cancer cell lines.**

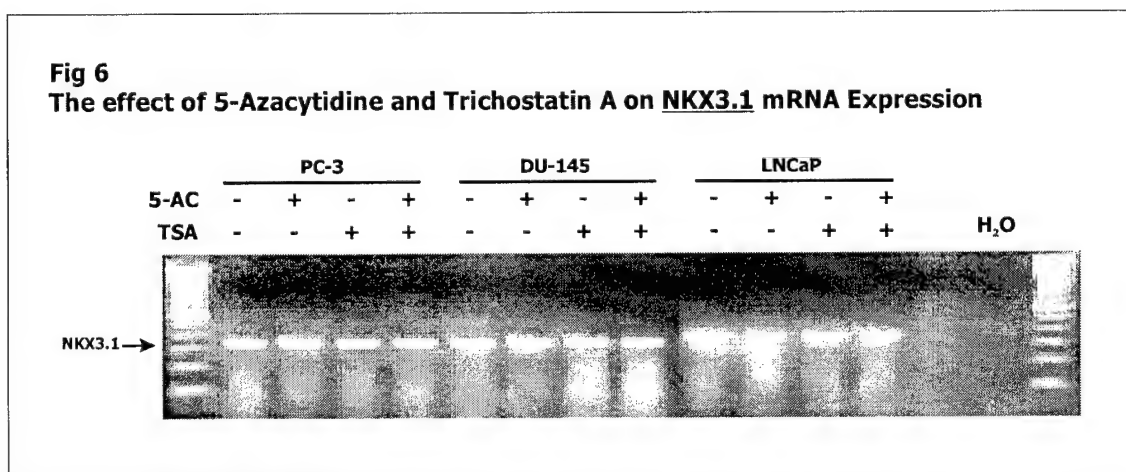
To determine whether control of NKX3.1 expression in cancer cell lines and tissues depended on gene methylation we treated cultured cells with methylation and histone deacetylase inhibitors and assayed *NKX3.1* mRNA expression by RT-PCR. We also analyzed protein expression

by western blotting. Prostate cancer cell lines PC-3, DU-145 and LNCaP were cultured routinely in Modified IMEM (GIBCO) with 5% fetal bovine serum at 37°C with 5% CO<sub>2</sub> atmosphere. To test inactivation and reactivation, cells were seeded at  $2 \times 10^5$  cells /T75 flask on day 0. After 24 hours, cells were treated with 5-aza-2'-deoxycytidine (5-AC) (Sigma) at a final concentration of 2  $\mu$ M, or with trichostatin A (TSA) (Wako) at a final concentration 200 ng/ml, or with both 5-AC and TSA for 24 to 72 hours, exchanging the medium every 24 hours. Identical volumes of solvent alone were used as controls. Genomic DNA was isolated by using Puregene DNA isolation Kit (Gentra Systems, Minneapolis, USA) with standard protocol. Total RNA was extracted by RNeasy Mini Kit (Qiagen Inc.Valencia, CA) and treated with DNase (Qiagen Inc.Valencia, CA) to limit the DNA contamination. The purified DNA or RNA was stored at -20 °C until use.

Figure 5 shows the results of these experiments. RT-PCR primer sequence for NKX3.1 were sense 5'agc cag agc cag agc cag agg 3', antisense: 5' ttg ggt ctc cgt gag ctt gag gtt 3'; RT-PCR product: 332 bp. There was no effect of 5-AC on *NKX3.1* expression in these cell lines.



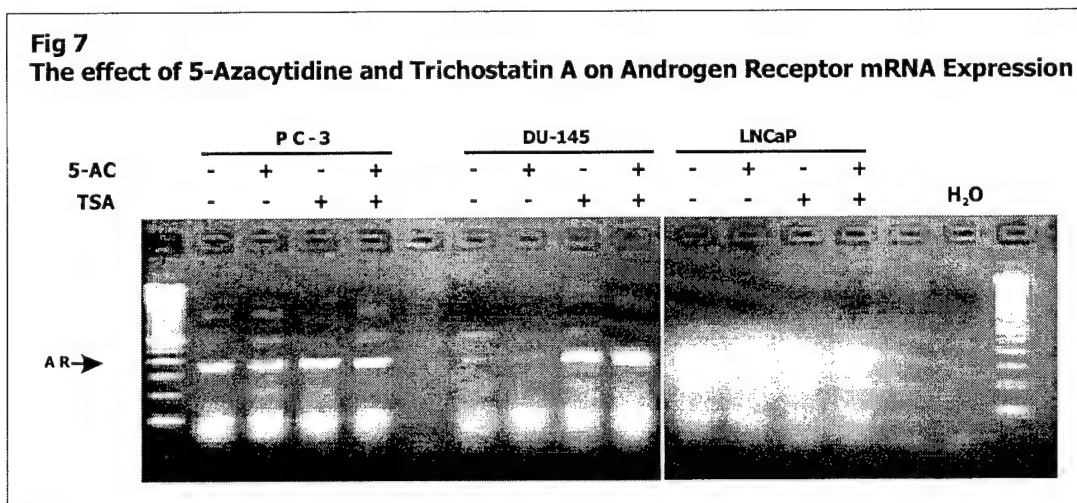
Addition of the histone deacetylase inhibitor, trichostatin A, at the 24 hour time point did not change the expression of *NKX3.1* as shown in Figure 6. We also found no effects of 5-AC and TSA on





NKX3.1 protein expression by western blotting in the three cell lines shown in Figure 6 (data not shown).

To be sure that the 5-AC and TSA were effective, we analyzed expression of androgen receptor (AR) in DU-145 cells after treatment with these agents. Figure 7 shows that consistent with published reports we were able to activate AR expression in DU-145 cells. The primers for AR were sense 5'gca tgg tga gca gag tgc cct atc 3', antisense: 5' tcc cag agt cat ccc tgc ttc at 3' to give an RT-PCR product of 365 bp (77,78).



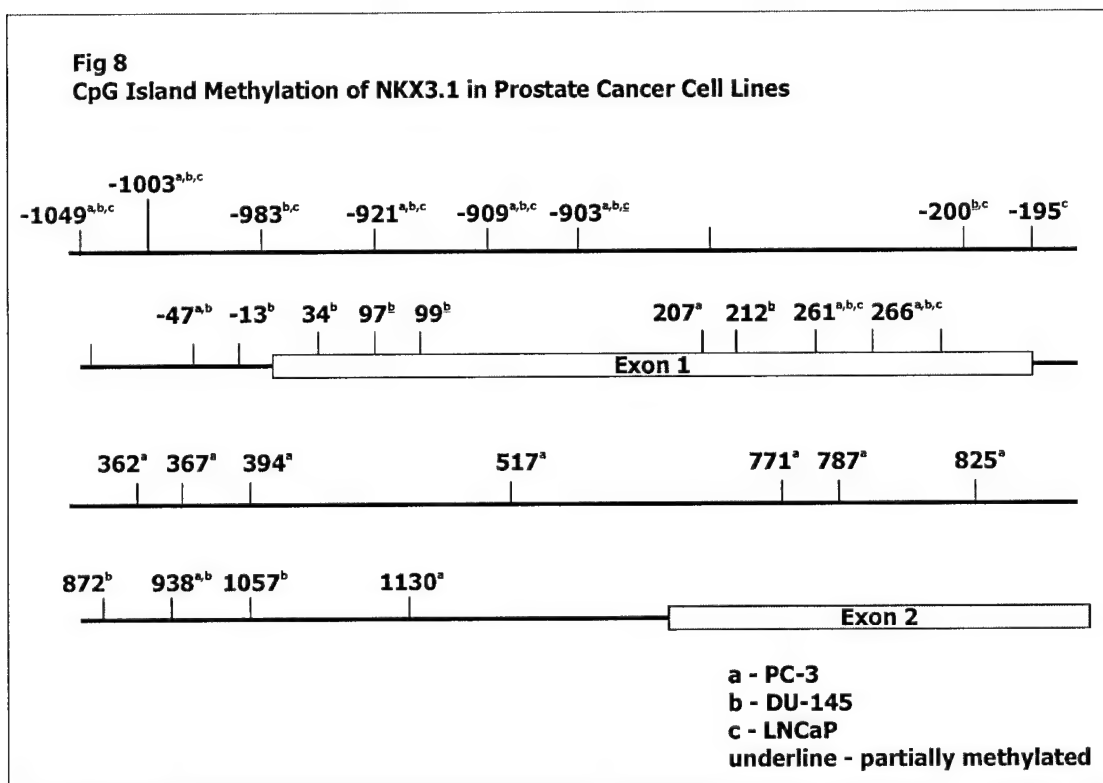
**D. Design and test primers for methylation-specific PCR.** – see section E.

**E. Perform methylation-specific PCR analysis of tissues with known NKX3.1 expression levels.**

To ask whether NKX3.1 was methylated in human prostate cancer compared to adjacent normal epithelial cells we developed primers to discriminate between methylated and unmethylated regions of the *NKX3.1* gene. Paraffin-embedded tissue was used to generate DNA after the block was marked for regions of prostate cancer and nonmalignant tissue. DNA (2 µg) in a volume of 50 µl was denatured in 0.3 M NaOH at 42°C for 10 min. Thirty micro liter of fresh prepared 10 mM hydroquinone and 520 µl of 3 M sodium bisulfite (Sigma) at pH 5.5 were added and mixed. The samples were covered with mineral oil and incubated at 50 °C for 16 hr. Modified DNA was desalted with the Wizard DNA clean-up system (Promega) according to the protocol provided by the manufacturer and resolved in 50 µl sterile water. 5.5 µl 3 M NaOH was added into 50 µl modified DNA solution and incubated at 37 °C for 10 min for denaturation, followed by ethanol precipitation with See DNA (Amersham Pharmacia Biotech) as co-precipitant. The DNA was resuspended in 20 µl sterile water, stored at -20 °C until use. For RT-PCR fifty ng of total RNA was used to a single RT-PCR reaction by using one step RT-PCR kit (Qiagen Inc.Valencia, CA, USA). The primers used for RT-PCR are as follows: NKX3.1: sense 5'agc cag agc cag agc cag agg 3', antisense: 5' ttg ggt ctc cgt gag ctt gag gtt 3'; AR: sense 5'gca tgg tga gca gag tgc cct atc 3', antisense: 5' tcc cag agt cat ccc tgc ttc at 3'.

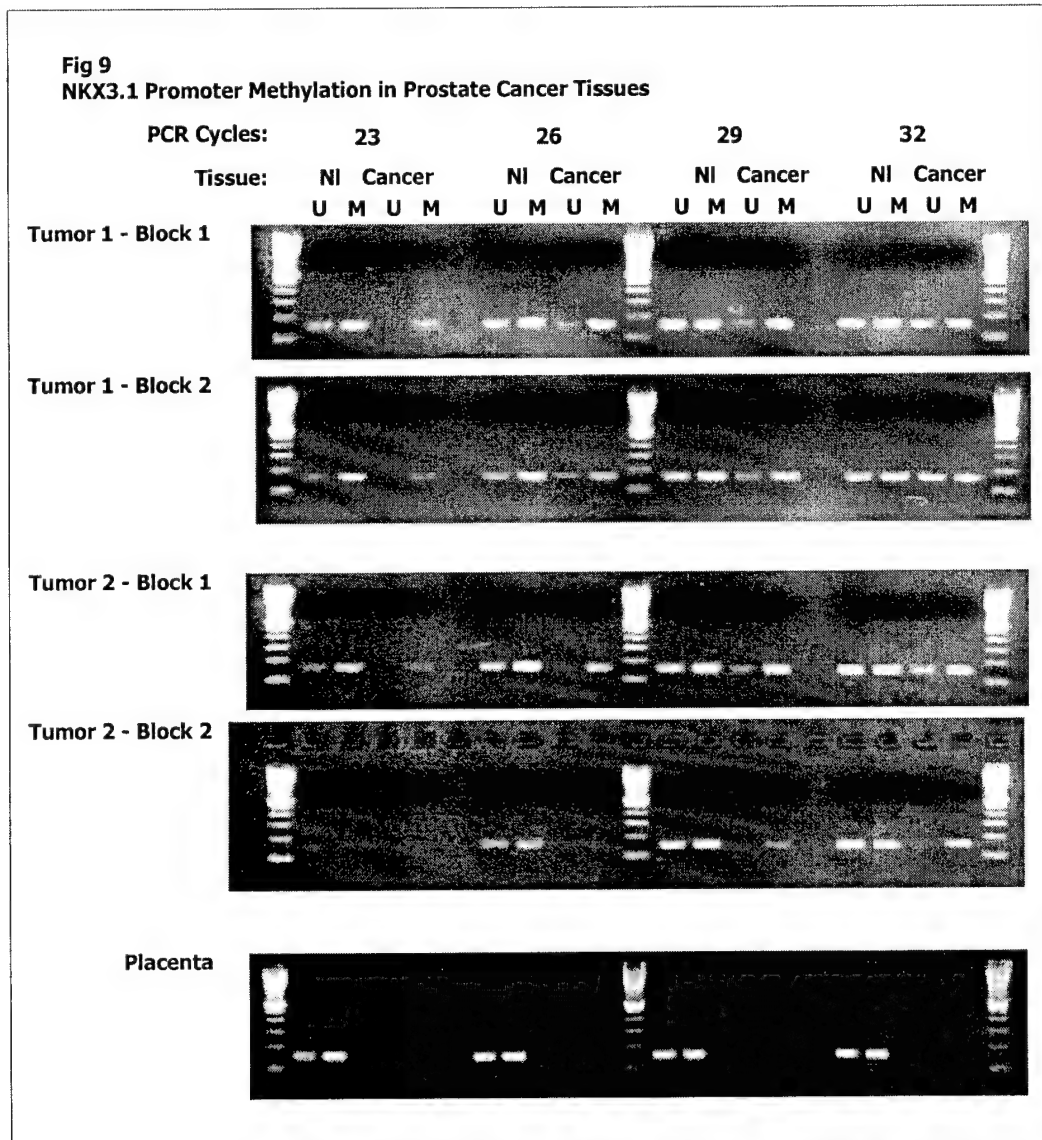
To define the methylation status of *NKX3.1* in prostate cancer cell lines we did PCR analysis of bisulfite-modified DNA (100 ng) was amplified by common or nestle PCR with NKX 3.1 specific primers: first nestle PCR primers for -1056 to -687: sense 5' GGT ATT TTG AGA GGT TAA GGT AGG AGG A 3' antisense 5'- CCT TTA TCC ACA ATA ACC TAT TAA CTT TCC TTC C -3'; second nestle PCR primers for -1056 to -687: sense 5'- AGG AAA TCG AGG TTG TAG TGA GTT ATG A -3', antisense: 5'- TCC CCA AAC ACA TAA CAT ACC ATC CT -3'; first nestle PCR primers for -687 to -233: sense: 5'- GGT ATG TTA TGT GTT TGG GGA GGA AGG A -3': antisense: 5'- CCC TAA AAA ATT CCT TCC CCA ATT CCC T -3'; second nestle PCR primers for

-687 to -233: sense: 5'- GGA AGG AAA GTT AAT AGG TTA TTG TGG ATA AAG GA -3', antisense: 5'- CTC CTT CCC CAA TTT CCT CTC CTT CT -3'. First nestle PCR primers for -233 to 323: 5'- GGA GAG GGA ATT GGG GAA GGA G -3'; antisense: 5'- TCC CTA CAC CCC AAA CTC TAA AAA TCC -3'. second nestle PCR primers for -233 to +323: 5'- AGG GAA TTG GGG AAG GAA TTT TTT AGG G -3', antisense: 5'- CCT TAA ACT ACC CCC CTT CCC C -3'. The primers for +301 to +656: sense: 5'- GGG GAA GGG GGG TAG TTT AAG G -3'; antisense: 5'- CTT TAC TAA TCA ACC CCT ACT TTA TCA CT -3'; The primers for +627 to +1172: 5'- AGT GAT AAA GTA GGG GTT GAT TAG TAA AG -3'; antisense: 5'- CCC TTA TCT CTT TAA TAA ATA AAC AAA CAA CCC -3'. The primers for detect the AR gene after bisulfite modification: 5'- tgg ttt agg aaa tta gga gtt att tag g -3'; 5'- antisense: tcc ctt cga ctc cta tac aac act a -3'. Genomic sequencing of bisulfite modified DNA was accomplished using purified PCR products as template for sequencing with the kit.



We observed that there was a higher degree of *NKX3.1* gene methylation in PC-3 and DU-145 cells than in LNCaP cells, perhaps due to the nascent activity of the gene in these cells. Because of this result we asked further whether there was differential methylation of the *NKX3.1* promoter in normal and cancer cells from prostate cancer tissues. For a preliminary analysis we chose to analyze the CpG island at -921 that was methylated in all three cell lines. The primers spanned the regions sense -1096 ~ -1066 (30 bp) and antisense -701 ~ -669 (32 bp) for the first reaction and for methylation-specific PCR: sense -1048 ~ -1024 (24 bp); antisense for -891 ~ -921 (30 bp); and antisense for nonmethylated-specific status -885 ~ -921 (36 bp). We found that this site was partially methylated in placental DNA and in nonmalignant regions of prostatectomy tissue blocks. On the other hand, there was preferential methylation of this site in prostate cancer taken from the same block. Note that we used DNA from two different blocks of the same tumor specimen to be sure that the results were not due to regional variations in the cancer specimen. Moreover, we obtain similar results with two different tissues (Figure 9). These results will allow us to expand our studies to sites that were methylated in DU-145 and PC-3, cells that do not express *NKX3.1*, but not methylated in

LNCaP, a cell that expresses *NKX3.1*. For example, in Figure 8 we would target CpG islands at –903, –47 and +938.



**F. Perform in situ FISH analysis of tissue microarray samples for *NEFL* and D8S7. Correlate the results with *NKX3.1* expression.**

Because very few tumors in the tissue microarray had complete loss of *NKX3.1* expression, we did not pursue these experiments. However, it became apparent from experiments with mice engineered for loss of one or both *Nkx3.1* alleles that haplinsufficiency was semidominant (3). Moreover, complete loss of *Nkx3.1* expression was seen in the earliest lesions of intraepithelial neoplasia in *Pten/Nkx3.1* compound deletion mice (7). We therefore sought to demonstrate that *NKX3.1* expression was reduced, though not absent in primary human prostate cancer. We have developed a quantitative assay for *NKX3.1* expression using immunofluorescence confocal microscopy to analyze levels of *NKX3.1* compared to expression of a stable nuclear protein, histone, chosen as an internal control. Nuclear staining for histone in prostate cancer tissues was anticipated to be uniform for the ~85% of prostate cancer cases with diploid DNA content (Bandalament, R. A., O'Toole, R. V., Young, D. C., and Drago, J. R. DNA ploidy and prostate-specific antigen as prognostic factors in clinically resectable prostate cancer. *Cancer*, 67: 3014-3023, 1991. In fact, we have shown that histone staining does vary with DNA content in cultured prostate cancer cell lines with aneuploid DNA content, but



it's variability is very narrow in prostate cancer tissue. The confocal assay was validated using the following cultured cell samples: LNCaP prostate cancer cells that express NKX3.1 in a androgen-regulated manner in the presence or absence of the synthetic androgen R1881; bladder cancer cell line TSU-Pr1, and a derivative TSU-Pr1(S11) cell line engineered to express NKX3.1.

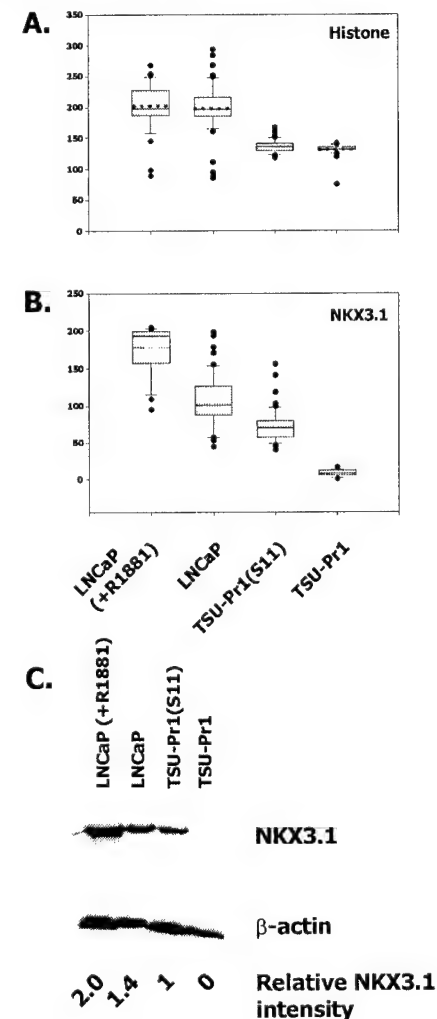
LNCaP, TSU Pr1 and TSU-Pr1(S11) cells were grown in IMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL) supplemented with 400 $\mu$ g/mL Gentamycin. LNCaP cells were grown in the presence or the absence of the synthetic androgen R1881 ( $10^{-9}$ M). Cells were transferred to 4-chamber slides, fixed for 15 minutes in 4% formaldehyde in PBS at room temperature, and permeabilized with 0.5% Triton X-100 in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, 0.1% NaN<sub>3</sub>, pH 7.6) for 3 minutes. Antigen retrieval was performed in citrate buffer using a standard steamer. Peroxide blocking was preformed with 3% peroxide in absolute methanol. Blocking of nonspecific binding sites was performed with Goat Serum in TBS (1:70) for 30min. Histone expression was detected with mouse monoclonal antihistone antibodies (1:1000) followed by secondary affinity-purified biotinylated horse antimouse antibody (Vector Laboratories Inc., Burlingame, CA) (1:200) and revealed with Texas red-avidin (Vector Laboratories) (1:200). NKX3.1 was detected with affinity-purified rabbit anti-NKX3.1 serum (1:1000)<sup>15</sup> and fluorescein-conjugated goat antirabbit antibody (Vector Laboratories Inc.) (1:200).

All images were collected on Olympus IX 70 confocal inverted microscope with laser scanning unit and 60X NA oil lense (Carl Zeiss). Samples were excited with Argon laser 488nm and Krypton laser 568nm. Areas imaged were randomly chosen while visualizing histone staining with Texas Red, common to all cells, to eliminate sampling bias. Intensity of obtained signal was calibrated to avoid saturation of signal, using Hue-Saturation-Intensity (HSI) channel of Fluoview 2.1 software (Olympus). Images were saved as 24-Bit color files.

Histone control staining was analyzed for ratio of mean of histone in tumor cells ( $H_T$ ) to mean of histone in normal cells ( $H_N$ ). If the ratio  $H_T:H_N$  is between 0.9 and 1.1 samples were dimmed evaluable and analyzed further. The ratio of NKX3.1 to histone expression was calculated for each of the selected cells. The mean of ratios was calculated for normal ( $r_n$ ) and cancerous areas ( $r_c$ ). Further analysis was done with log of mean values for cancer and normal areas ( $\log(r_c)$  and  $\log(r_n)$ , respectively) for each sample. The difference in log ratio values will be calculated:  $\log(r_c) - \log(r_n)$ .

The assay was validated with cultured cells as shown in Figure 10. Histone staining was higher in LNCaP cells then TSU-Pr1 and TSU-Pr1(S11) cells. This correlated with DNA content in LNCaP cells measured by flow cytometry of propidium iodide-stained cells. LNCaP cells had ploidy index of 1.8 times normal and TSU-Pr1 cells and TSU-PR1(S11) cells had a ploidy index of 1.5. Note that the ratio of histone expression in LNCaP:TSU-Pr1 was 1.46, and the ratio of ploidies is 1.2. NKX3.1 staining was the highest in LNCaP cells exposed to the

**Fig 10**  
**Confocal Assay of NKX3.1**  
**Expression in Cultured Cells**



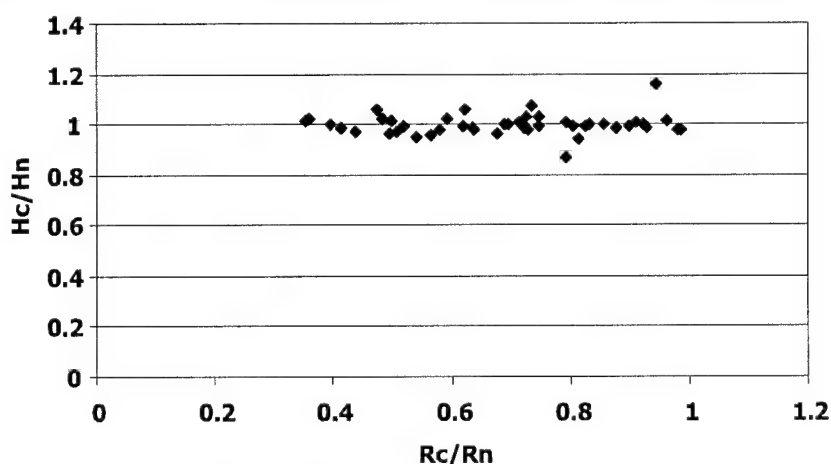
androgen R1881 (mean = 175), followed by LNCaP cells (mean = 100), then TSU-Pr1(S11) (mean = 70) and was close to background in TSU-Pr1 (mean = 0). The confocal assay of NKX3.1 expression correlated well with the results of the western blot shown in Figure 10C.

Forty-six paraffin-embedded prostate cancer specimens were sectioned in parallel 4- $\mu$ m sections with a Leitz microtome. The sections were then deparaffinized with standard xylene and hydrated through graded alcohols into water. One section from each tissue block was stained with hematoxylin and eosin (H&E) and covered with a coverslip. The remaining sections were stored at room temperature for immunofluorescent staining. Areas of tumor and nonmalignant epithelial cells were marked on H&E sections.

Obtained images were analyzed using the MetaMorph software (Universal Imaging Corporation). Computer assisted tracing of nuclei on randomly chosen cells (n=50) from each cell type on the slide was done, while visualizing cells for Texas Red (histone) staining to decrease sampling bias. Intensity of FITC (NKX3.1) and Texas Red (Histone) staining was calculated using Average intensity measuring tool of the software. Data was expressed in pixels and logged to Excel 2000 sheets for further statistical analysis. Histone staining in malignant and normal epithelial cells was largely invariant and found to be within 10% of unity in 44 of 46 tumors. Whereas the intensity of histone staining was measured in a narrow range across all malignant and normal epithelial cells, staining for NKX3.1 was found to vary over a broad range and was almost always lower in malignant cells than normal cells. We found that NKX3.1 expression was reduced to 80% or less of control in 32/46 samples and to 90% or less in 39/46 samples (Figure 11).

We plan to perform interphase fluorescent *in situ* hybridization (FISH) with the NKX3.1 promoter region clone described above and a chromosome 8 centromere probe to identify which of the tumors also underwent chromosome 8p21 loss of heterozygosity.

**Fig 11**  
**Normalized NKX3.1 Expression in Prostate Cancer Tissues**



### **3. Determine the mechanism by which NKX3.1 alters the cell death response in TSU-Pr1 prostate cancer cells transfected with control plasmid or with NKX3.1. (Years 1-2)**

Our preliminary data had indicated that NKX3.1 expression conferred increased sensitivity to TSU-Pr1 cells for radiation induction of cell death. We conducted a large series of experiments to explore this finding as was outline in the Statement of Work. However, when we had difficulty getting consistent results we repeated the initial experiments and found that the observation was not reproducible. We performed transient transfection of TSU-Pr1 bladder cancer cells and PC-3 prostate cancer cells and compared cell death induction in the transfected and untransfected cells from the same culture. PC3 and TSU-Pr1 cells were maintained in Modified Improved MEM (Life

Technologies, Inc.) supplemented with 5% fetal bovine serum. Cells were plated at  $1-2 \times 10^5$  cells/well in 6 well plates. Cells were transfected 24 hours after plating with expression plasmids for green fluorescent protein (GFP) and either control vector, NKX3.1, or NKX3.1 $\Delta$ (184-234) using Lipofectamine Plus according to manufacturer's protocol (Life Technologies, Inc.). Each transfection reaction contained 0.2 ug of test plasmid and 0.2ug GFP plasmid. The medium was changed to Modified Improved MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum prior to 20Gy irradiation. Forty-eight hours after irradiation cells were sorted by fluorescent intensity in a FACStar Plus sorter (Becton Dickinson). Both GFP-positive and GFP-negative cells were analyzed by in situ end-labeling assay.

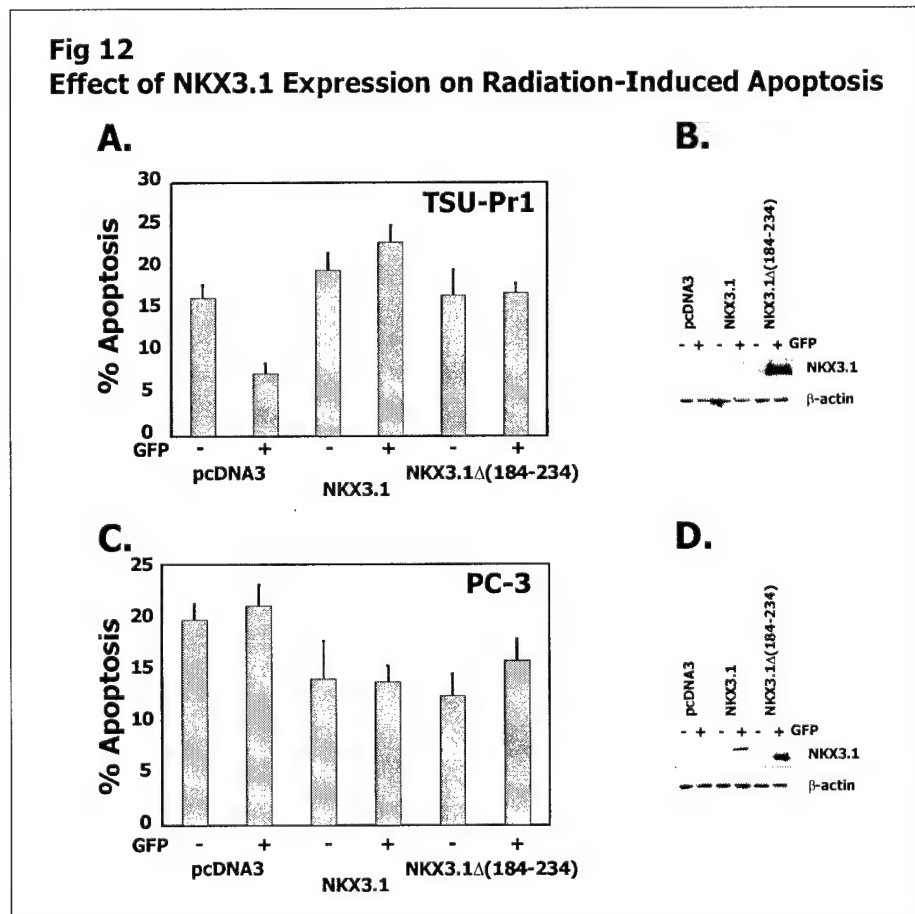
As shown in Figure 12 we found no substantial differences in the induction of apoptosis between the GFP+ and GFP- populations. The figure also shows that we analyzed expression of NKX3.1 by western blotting and showed that sorting for GFP expression did segregate cells into those that expressed the transfected plasmids and those that did not. Figure 12A and B shows results for TSU-Pr1 cells and those in Figures 12C and D for PC-3 cells.

#### 4. Determine if NKX3.1 expression alters in vivo tumor growth and tumor response to cytotoxic therapy.

**Effect of etoposide or doxorubicin on xenografts from control and NKX3.1-expressing TSU-Pr1 clones. To use 200 mice total, 5 mice per cell line for etoposide, and 5 mice per cell line for sham-treated animals. (Year 2)**

##### A. Inoculate mice with NKX3.1 + and – derivative TSU-Pr1 clones

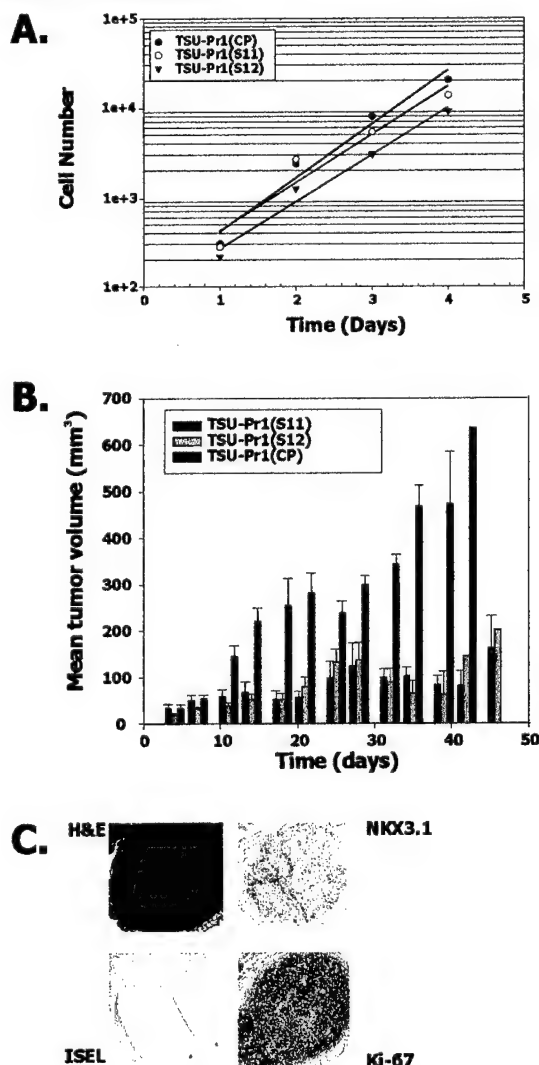
Initially we examined the growth effects of NKX3.1 expression on cultured cells. The TSU-Pr1 cell line was chosen for these experiments at a time when they were believed to be of prostatic origin



(79). Recently they have been shown to be of bladder origin (80). However, the relevance of this model to determining the tumor suppressor properties of NKX3.1 is not affected by our revised understanding of the cell of origin. Like androgen-independent prostate cancer cell lines these cells make very low levels of *NKX3.1* mRNA, no detectable protein by immunohistochemistry and do not express prostate-specific antigen or androgen receptor. They grow vigorously in culture and as nude mouse xenografts and are appropriate for testing tumor suppressor properties of genes. In situ end labeling for apoptotic cells was done as previously described (81,82). Stable transfectants were derived from a pool of antibiotic-resistant TSU-Pr1 [TSU-Pr1(CP)] cells transfected with pcDNA3 (Invitrogen) and from a series of clones transfected with pcDNA3 containing full-length *NKX3.1* cDNA (1). To retain expression of NKX3.1 cells had to be passed in 400µg/ml G418. Expression was confirmed by periodic western blotting with anti-NKX3.1 antibody (6). Two independent clones, TSU-Pr1(S11) and (S12) were used in these experiments. Cell counts for in vitro growth were done by hemacytometer. Analysis of the in vitro growth data was done by plotting a regression line through the log phase growth points using Sigmaplot 2000. Stable expression of full-length NKX3.1 in clones of TSU-Pr1 cells diminished in vitro growth and increased doubling time by about 15% (Figure 13A and Table 5). Expression of NKX3.1 in TSU-Pr1 stable transfectants has been previously demonstrated by western blotting (6).

To examine the effects of NKX3.1 expression on TSU-Pr1 tumor formation male athymic nude mice (Taconic Labs) were injected subcutaneously with 0.15 ml of  $0.5 \times 10^7$  cells suspended in serum-free medium. Cells had been washed twice in serum-free medium after harvest from culture. Three-dimensional tumor measurements were made every three-four days starting seven days post injection. Tumors were harvested for histochemical analysis by standard techniques (83). Expression of NKX3.1 in TSU-Pr1 stable transfectants has been previously demonstrated by western blotting (6). Growth of two NKX3.1-expressing TSU-Pr1 cell lines as xenografts in nude mice was markedly attenuated compared to TSU-Pr1(CP) cells (Figure 13B). Tumor sizes are plotted on a linear scale because most tumors that exceeded approximately 100 mm<sup>3</sup> demonstrated central necrosis and therefore tumor volume was not an accurate estimate of cell number. The slope of the growth for TSU-Pr1(CP) tumors was four times the rate of growth of the NKX3.1-expressing clones. TSU-Pr1(S11) cells also displayed a lower rate of tumor take than the other two cell lines. Tumor takes were determined by microscopic analysis of the tumor cell inoculation site. Several of the TSU-Pr1(S12) tumors harvested between 21 and 45 days were found histologically to be present as microscopic ~0.1mm nodules embedded in

**Fig 13**  
**Effect of NKX3.1 Expression on Growth and Tumorigenesis**



the dermis. These were scored as tumor takes even though they were not macroscopic tumors. TSU-Pr1(CP) cells gave only macroscopic tumors and showed none of these intradermal microscopic nodules.

Although the TSU-Pr1(S11) and TSU-Pr1(S12) tumors were consistently smaller than the counterpart control tumors grown in the same animals, we were unable to detect differences in histology appearance between the two kinds of tumors. All tumors, both control and NKX3.1-transfected, were poorly differentiated carcinomas (Figure 13C). Differences in tumor growth could not be explained by differences in apoptotic index. The number of apoptotic cells outside of necrotic areas was counted for each tumor. We could not discern differences in apoptotic cell number between the cell lines (Table 5). The NKX3.1-transfected, but not the control cells, were seen to express nuclear NKX3.1 in 20-30% of the cells in vivo. Lastly, tumors from both the control and transfected cells had similar levels of staining with antibody to Ki-67 (MIB1), an indicator for proliferation (Figure 13C and Table 5). Analysis of collections of erythrocytes in the tumors, an index of angiogenesis, also showed no differences between control and NKX3.1-expressing cells, suggesting that there were no major differences in angiogenesis between the two kinds of tumors (not shown). Therefore it appeared that NKX3.1 expression conferred a subtle depressive effect on TSU-Pr1 cell growth that translated into decreased capacity for tumorigenesis.

**Table 5**  
**Growth Properties of TSU-Pr1 clones**

Cell Line	In vitro $t_{1/2}$ (days) <sup>a</sup>	Tumors/ Injection sites <sup>b</sup>	% NKX3.1 Expressing cells <sup>c</sup>	Apoptotic cells per tumor <sup>d</sup>	%Ki-67- Positive Cells <sup>e</sup>
TSU-Pr1(CP)	0.50	22/24	0	8.2±9.6	65±10
TSU-Pr1(S11)	0.57	14/24	30±8	6.6±6.2	63±11
TUS-Pr1(S12)	0.56	22/24	24±14	4.0±5.0	65±8

<sup>a</sup>The  $t_{1/2}$  is calculated from the data in Figure 1A.

<sup>b</sup>Tumors/injection sites shows the number of tumor takes in the numerator and the total number of sites inoculated with tumor cells. Tumor take is defined in the text.

<sup>c</sup>The fraction of cells expressing NKX3.1 were determined by counting cells with nuclear immunohistochemical staining for NKX3.1 in a minimum of 300 total cells counted.

<sup>d</sup>Apoptotic cells per tumor were determined by counting all single-cells staining in the entire tumor section after ISEL of a tumor section (83).

<sup>e</sup>KI-67 positive cells were determined by counting cells that stained for KI-67 in a minimum of 300 total cells counted.

## **B. Treat with intraperitoneal etoposide and observe tumor responses.**

Because the expression of NKX3.1 in TSU-Pr1 cells by itself inhibited tumor growth even though the effects on in vitro growth were limited, this element of the Statement of Work was not feasible and was not pursued.

**C. If necessary, repeat with doxorubicin or irradiation.**

Because the expression of NKX3.1 in TSU-Pr1 cells by itself inhibited tumor growth even though the effects on in vitro growth were limited, this element of the Statement of Work was not feasible and was not pursued.

## KEY RESEARCH ACCOMPLISHMENTS:

- Development of the KPNW prognostic cohort and construction of tissue microarray
- Determination that NKX3.1 promoter was partially methylated in prostate cancer cells
- Demonstration that NKX3.1 was a tumor suppressor in a xenograft model
- Determination that the *NKX3.1* promoter is not directly affected by androgen.
- Determination that prostate-specific expression of *NKX3.1* requires a large region of the promoter
- Demonstration that NKX3.1 expression was down regulated in a large fraction of primary prostate cancer tissues

## REPORTABLE OUTCOMES:

- Tumor suppressor properties of NKX3.1
- Down regulation of NKX3.1 expression in primary prostate cancer
- Methylation of NKX3.1 promoter in prostate cancers

## CONCLUSIONS:

*NKX3.1* is a gene with prostate-specific expression that codes for a protein that regulates growth suppression and differentiation in the prostate. Loss of 8p21 and *NKX3.1* heterozygosity in the majority of human prostate cancers results in reduced protein expression that, in mouse models, can cause loss of growth control in prostatic epithelium. We and others have shown that *NKX3.1* has growth and tumor suppressor properties in model systems. This may be one of the first events in human prostate carcinogenesis and leads us to contend that *NKX3.1* is the prostate cancer gatekeeper gene. Control of NKX3.1 expression in the prostate is complex. In both mouse and human tumors mRNA expression is not down regulated at times when NKX3.1 protein expression is lost. However, we have now shown that the *NKX3.1* promoter is differentially methylated in prostate cancer cells and adjacent normal cells, suggesting that alteration of RNA expression may also play a role in modulating NKX3.1 expression in the prostate.

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# DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1

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## ABSTRACT

**NKX3.1 is a member of the NK class of homeodomain proteins and is most closely related to *Drosophila* NK-3. NKX3.1 has predominantly prostate-specific expression in the adult human. Previous studies suggested that NKX3.1 exerts a growth-suppressive effect on prostatic epithelial cells and controls differentiated glandular functions. Using a binding site selection assay with recombinant NKX3.1 protein we identified a TAAGTA consensus binding sequence that has not been reported for any other NK class homeoprotein. By electromobility shift assay we demonstrated that NKX3.1 preferentially binds the TAAGTA sequence rather than the binding site for Nkx2.1 (CAAGTG) or Msx1 (TAATTG). Using mutated binding sites in competitive gel shift assays, we analyzed the nucleotides in the TAAGTA consensus sequence that are important for NKX3.1 binding. The consensus binding site of a naturally occurring polymorphic NKX3.1 protein with arginine replaced by cysteine at position 52 was identical to the wild-type binding sequence. The binding affinities of wild-type and polymorphic NKX3.1 for the TAAGTA consensus site were very similar, with values of 20 and 22 nM, respectively. Wild-type and polymorphic NKX3.1 specifically repressed transcription of luciferase from a reporter vector with three copies of the NKX3.1-binding site upstream from a thymidine kinase promoter. The data show that among NK family proteins NKX3.1 binds a novel DNA sequence and can behave as an *in vitro* transcriptional repressor.**

## INTRODUCTION

Homeobox genes encode proteins that contain highly conserved DNA-binding regions called homeodomains. Homeodomain proteins, or homeoproteins, bind to specific DNA sequences and function as transcription factors that regulate eukaryotic development with spatial and temporal specificity (1). NKX3.1 is a novel homeoprotein that belongs to the *Drosophila* NK class of homeodomain proteins. NKX3.1 is most closely related to *Drosophila* NK-3 by virtue

of 78% sequence homology to the NK-3 homeodomain region (2–4). Murine Nkx3.1 has 100% sequence homology to the human NKX3.1 homeodomain region and shares 90% sequence homology overall (3–6).

Expression of murine *Nkx3.1* in the developing embryo and fetus has been detected in a variety of tissue types, including mesoderm, vascular smooth muscle, epithelium and regions of the central nervous system (7–9). However, in the adult mouse *Nkx3.1* expression is androgen regulated (5) and restricted primarily to the prostate and the bulbourethral gland (6,9). NKX3.1 expression in the adult human is also androgen regulated and localized predominantly in the prostate, with low levels also detected in the testis (3,4).

NKX3.1 has been mapped to human chromosome 8p21, a locus that is frequently deleted in prostate cancer (10–13). Therefore, NKX3.1 is a candidate tumor suppressor gene. However, no cancer-specific mutations of the NKX3.1 coding region were found in human prostate cancer samples (10). Nevertheless, the potential for NKX3.1 to exert a differentiating and growth suppressing effect on prostatic epithelium was confirmed by targeted gene disruption of *Nkx3.1* in mice (9). Deletion of either one or both copies of *Nkx3.1* resulted in prostatic epithelial hyperplasia and dysplasia that increased in severity with age. Homozygous deletion of *Nkx3.1* caused defective prostate ductal morphogenesis and decreased seminal protein production. Loss of NKX3.1 protein expression may be important in prostate cancer pathogenesis. We have recently shown that ~40% of human prostate cancer samples had diminished expression of NKX3.1 compared to adjacent normal epithelium (Bowen *et al.*, submitted for publication).

Genetic analysis indicated that ~10–15% of human DNA samples contain a polymorphic NKX3.1 gene characterized by the presence of a C→T polymorphism at nucleotide 154 (10; our unpublished data). The polymorphism resulted in the substitution of arginine by cysteine at codon 52, located N-terminal to the homeodomain. To date, a phenotype has not been identified for the polymorphic protein.

NK-2 class homeoproteins preferentially bind DNA sequences with a CAAG core sequence (14,15), while other NK class homeoproteins preferentially bind TAAT core sequences (16,17). The optimal DNA binding site for *Drosophila* NK-3 or its mammalian homologs has yet to be defined. Using a binding site selection assay, we identified the *in vitro* DNA binding site of NKX3.1. Electromobility gel shift assays were used to analyze binding affinity and nucleotides in the consensus site that are important for NKX3.1 binding. By

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inserting three copies of the NKX3.1-binding site into a luciferase reporter vector, we investigated the transcriptional function of NKX3.1 using reporter gene analysis. Finally, the DNA binding and transcriptional activities of wild-type and polymorphic (R52C) NKX3.1 were compared to determine if differences existed between the proteins.

## MATERIALS AND METHODS

### Expression and reporter plasmid construction

Full-length NKX3.1 cDNA was synthesized by PCR amplification of full-length wild-type human NKX3.1 cDNA obtained from a normal prostate cDNA library (3). Amplification was carried out for 30 cycles (94°C for 30 s, 62°C for 30 s and 72°C for 60 s) using a Perkin Elmer temperature cycler (Perkin Elmer, Norwalk, CT). The primers (Gibco BRL, Rockville, MD) used in the amplification incorporated *Eco*RI and *Xho*I restriction sites for directional cloning of the amplified DNA into expression vectors. The forward PCR primer has the sequence 5'-CGG-GATCCGAATTCATGCTCAGGGTTCGGAGCCGC-3' and the reverse primer has the sequence 5'-GGGCCTCGAGTCTAGAGTTACCCAAAAGCTGGGCTCCA-3'. The amplified DNA was cloned into a pCRII-Topo vector (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer. The DNA was digested with *Eco*RI and *Xho*I restriction enzymes and the fragment representing NKX3.1 cDNA was excised from a 1% agarose gel. The DNA was purified using a QiaexII DNA isolation kit (Qiagen, Valencia, CA) and the resulting fragment was directionally cloned into the *Eco*RI and *Xho*I restriction sites of plasmid pcDNA3 (Invitrogen) for mammalian protein expression. NKX3.1 cDNA was also cloned into the *Eco*RI and *Sal*I sites of the pMAL-C2G vector (New England Biolabs, Beverly, MA) for bacterial protein expression. Wild-type NKX3.1 cDNA was altered using a Stratagene Quikchange Site-Directed Mutagenesis kit following the manufacturer's protocol (Stratagene, La Jolla, CA). R52C NKX3.1 was generated by mutating the nucleotide sequence of codon 52 of wild-type NKX3.1 cDNA from CGC to TGC, thereby changing the corresponding amino acid from arginine to cysteine. The mutant NKX3.1 cDNA was sequenced entirely to ensure that no additional mutations were generated.

Luciferase reporter vectors were constructed by ligating an insert with partial *Xho*I ends containing three copies of TAAGTA or CACGTG into the *Xho*I site of pT109, which contains a herpes simplex virus thymidine kinase (TK) promoter upstream of a firefly luciferase gene. The sequence of one strand of the TAAGTA insert (binding sites underlined) containing a partial *Xho*I site was 5'-TCGATATTAAGTAT-AGGATTAAGTATAGGGATTAAGTAT-3' and the sequence of one strand of the CACGTG insert was 5'-TCGATATCACGTGTAGGATCACGTGTAGGGATCACGTGT-3'. The orientation of the TAAGTA insert was confirmed by sequence analysis.

### Bacterial protein expression and purification of recombinant NKX3.1

Plasmids containing wild-type or R52C NKX3.1 cDNA inserted into pMAL-C2G were used to transform competent *Escherichia coli* strain BL21 using standard techniques.

Transformants were used to inoculate 10 ml of Luria Bertani (LB) medium and the cultures were incubated with shaking at 220 r.p.m. overnight at 37°C. This culture was used to inoculate 1 l of LB medium containing 0.2% glucose, followed by incubation at 37°C with shaking at 220 r.p.m. until the optical density at 600 nm reached 0.6. Isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration of 0.3 mM and the culture was incubated for an additional 60 min. Cells were harvested by centrifugation and frozen at -80°C. Approximately 1 g of frozen cells were thawed on ice and resuspended in 10 ml of buffer A [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT)]. Suspended cells were lysed by sonication and a cell-free extract was prepared by centrifugation. The cell-free extract was loaded onto an amylose column (New England Biolabs) and washed with buffer A containing 10 mM maltose. Protein purity was demonstrated by 12% SDS-PAGE and purified protein concentration was determined by the Bradford assay (18).

### Selection and amplification binding assay

A selection and amplification binding (SAAB) assay was performed essentially as described for murine Nkx2.5 (15), with modifications. Briefly, 0.5 pmol of a 15 bp random sequence flanked by 20 bp regions of non-random sequence was radiolabeled with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. The radiolabeled probe was incubated with 25 pmol of purified wild-type or R52C NKX3.1 fusion protein in binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 5% glycerol, 5% sucrose, 0.1% Nonidet P-40, 0.1  $\mu$ g BSA, 1  $\mu$ g poly(dI-dC) and 5 mM DTT]. Unbound probe was separated from protein-bound DNA by native 8% PAGE in 0.5 $\times$  TBE buffer. Protein-bound DNA was detected by autoradiography and the bands representing protein-DNA complexes were excised from the gel. The DNA was eluted from the gel slice overnight at 37°C in elution buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS). Eluted DNA was PCR amplified using primers complementary to the 20 bp non-random flanking sequences and purified by native 12% PAGE in 1 $\times$  TBE buffer. The purified DNA was radiolabeled and used as a probe for another round of SAAB for a total of five rounds. The amount of protein was reduced to 5 pmol for the fourth and fifth rounds of SAAB. Following the final PCR amplification and gel purification, the 55 bp fragment was cloned into pCRII-TOPO using a TOPO-TA cloning kit (Stratagene). The inserts were sequenced on an ABI 377 Nucleotide Sequencer (Perkin Elmer).

### Electromobility gel shift assay

Double-stranded DNA used as probe or competitor was prepared by annealing equimolar amounts of complementary oligonucleotides with flanking partial *Bam*HI ends and filled-in with Klenow polymerase. The double-stranded DNA used as probe was radiolabeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. The labeled probe (1 pmol) was added to wild-type or R52C NKX3.1 (125 ng) in binding buffer to a final volume of 20  $\mu$ l. Competition binding experiments were performed essentially as described by Amendt *et al.* (19) by including unlabeled competitor DNA concentrations of 50, 250 and 500 nM. Competitor DNAs were preincubated with protein at room temperature for 30 min prior to addition of radiolabeled



probe. The nucleotide sequence of one competitor strand is listed, where used, in the appropriate figure. For dissociation constant ( $K_d$ ) measurements,  $1 \times 10^{-10}$  M radiolabeled probe was incubated with protein concentrations ranging from 0.5 to  $200 \times 10^{-9}$  M for 1 h prior to electrophoresis. Protein-DNA complexes were separated from unbound probe by native 8% PAGE at 175 V for 1 h at ambient temperature in  $0.5 \times$  TBE buffer following pre-electrophoresis of the gel at 200 V for 60 min. Following electrophoresis, a Molecular Dynamics PhosphorImager Screen (Molecular Dynamics, Sunnyvale, CA) was exposed to the dried gel. A Molecular Dynamics PhosphorImager and ImageQuaNT software (Molecular Dynamics) were used in the quantitative analyses. Binding competition was determined by calculating the ratio of bound to free probe normalized to the absence of competitor DNA. Dissociation constants were calculated from the binding data by plotting bound protein versus free protein and using the expression  $K_d = [D][P]/[DP]$  in a non-linear least squares analysis of the data. Free DNA is represented by [D], [P] is the free protein concentration and [DP] is the concentration of DNA-protein complex. Competition binding and dissociation constant measurements were performed at least three times and representative gel shift assays from the competition binding experiments are shown.

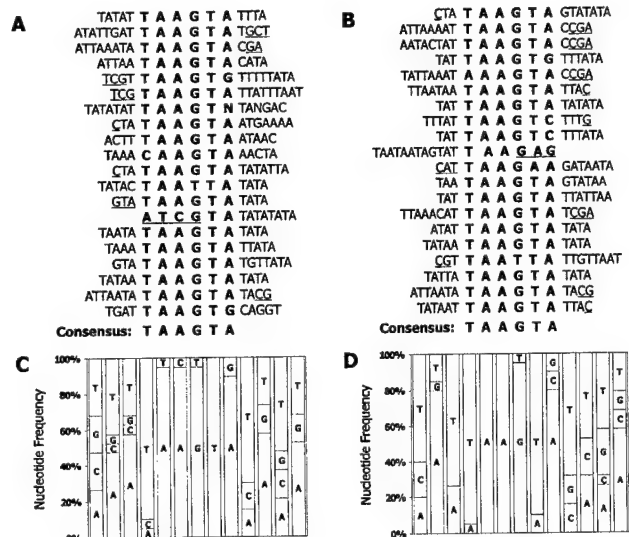
### Cell culture and reporter gene assays

The prostate carcinoma cell line TSU-Pr1 (20) was cultured in Improved Minimal Essential Medium (Gibco BRL) supplemented with 5% fetal calf serum. Cells were transiently transfected using  $6 \times 10^4$  cells/well in a 24-well plate with 250 ng reporter plasmid, 250 ng expression plasmid and 1 ng CMV-*Renilla* plasmid in the presence of 2  $\mu$ g LipofectAMINE 2000 (Gibco BRL) following the manufacturer's protocol. Cells were lysed 48 h after transfection and the lysate was assayed for firefly and *Renilla* luciferase activities using Dual Luciferase Reporter Assay reagents (Promega, Madison, WI). The data represent the average of three separate experiments normalized by *Renilla* luciferase activity.

## RESULTS

### Determination of the NKX3.1 consensus binding sequence by SAAB assay

The SAAB assay originally described by Blackwell and Weintraub (21) with modifications by Chen and Schwartz (15) was used to determine a consensus DNA binding sequence for bacterially expressed wild-type and R52C NKX3.1 fusion proteins. Purified proteins were used to select preferred binding sequences from a random pool of double-stranded DNA. Five cycles of binding selection were used to isolate optimal binding sequences and the enriched double-stranded DNA was cloned and sequenced. Alignment of the 20 selected sequences each for wild-type (Fig. 1A) and R52C NKX3.1 (Fig. 1B) revealed an identical consensus sequence of 6 nt (TAAGTA) with an individual nucleotide frequency of occurrence ranging from 78 to 100% (Fig. 1C and D). The NKX3.1 consensus sequence is similar, but not identical, to the binding sequences of other NK-2 class homeoproteins such as the CAAGTG site for Nkx2.1 (14) and TNAAGTG for Nkx2.5 (15). The consensus sequence for NKX3.1 is also similar to the TAAGTG consensus binding



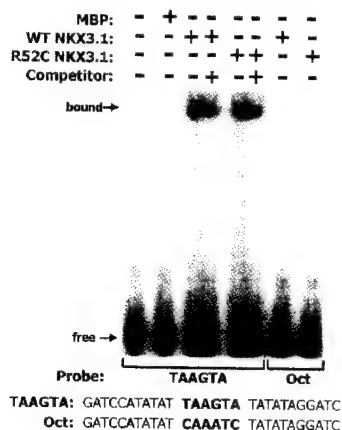
**Figure 1.** Determination of the NKX3.1 consensus DNA binding sequence. Sequences selected by (A) recombinant wild-type or (B) R52C NKX3.1 following five rounds of SAAB are listed. Underlined nucleotides represent non-random sequence. Nucleotides shown in bold represent those used in determination of the consensus binding sequence. The overall consensus binding sequence for each protein is listed below the aligned sequences. The percentage frequencies of each nucleotide for sequences selected by (C) wild-type NKX3.1 and (D) R52C NKX3.1 are shown.

sites of human Hox11 (22) and salmon Isl-2 (23), members of the Hox and LIM families of homeoproteins, respectively. The selected sequences also contained the TAAT motif recognized by a majority of homeodomain proteins with a characterized DNA-binding site (1). The TAAT motif was present at least once in 88% of the combined wild-type and R52C selected sequences. In similar binding assays Nkx2.5 and Isl-2 also selected sequences containing TAAT, in addition to their consensus binding sites (15,23).

Specific binding of wild-type and R52C NKX3.1 to the TAAGTA site was demonstrated by gel mobility shift (Fig. 2). Maltose-binding protein lacking the NKX3.1 moiety did not bind the TAAGTA sequence, suggesting that protein-DNA interactions were specifically due to the NKX3.1 segment of the fusion protein. Binding specificity was confirmed by competition with a 5-fold molar excess of unlabeled NKX3.1 consensus sequence, which competed for the TAAGTA probe. An Oct POU-1 homeodomain binding site was included as a control for non-specific DNA binding. The Oct site contains a CTAAAC binding site rather than the preferentially bound TAAGTA sequence. Neither protein bound the Oct probe.

### Comparison of wild-type NKX3.1 binding to the NKX3.1, Nkx2.1 and Msx1 binding sites

The results of the SAAB assay indicated that NKX3.1 preferentially bound a TAAGTA sequence. Using a competitive binding assay, we compared wild-type NKX3.1 binding to the TAAGTA site with binding to the Nkx2.1 and Msx1 DNA binding sites. The Nkx2.1 sequence (14) has a CAAG core

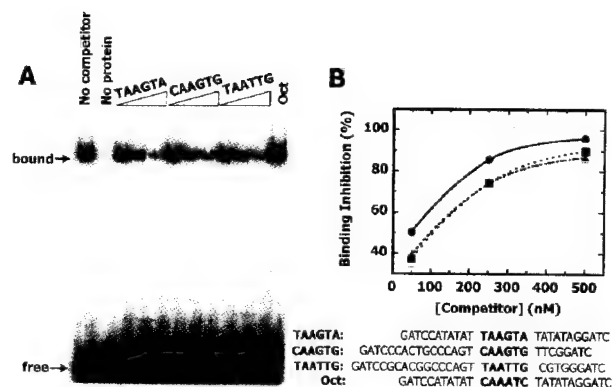


**Figure 2.** NKX3.1 specifically binds to the TAAGTA consensus binding site. Binding of maltose-binding protein, wild-type NKX3.1 and R52C NKX3.1 to a radiolabeled probe containing either the NKX3.1 consensus sequence (TAAGTA) or an Oct-1 binding site (CTAAAC) was analyzed by gel mobility shift. Free probe and protein-bound complex are indicated with arrows. Where indicated, a 5-fold molar excess of unlabeled TAAGTA sequence was included as competitor. Nucleotide sequences of the probes and competitor are listed at the bottom of the figure.

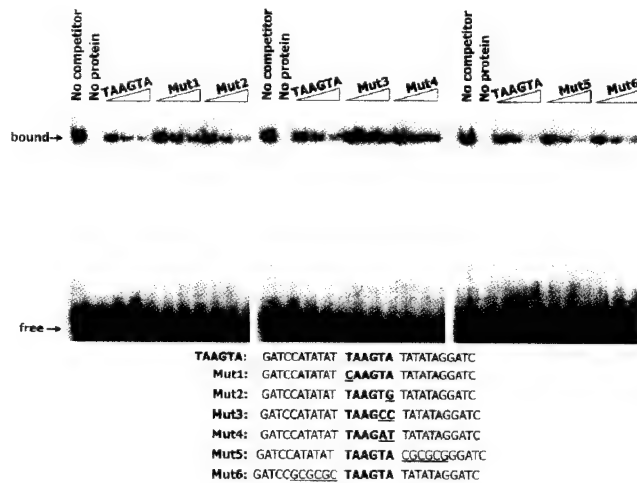
binding site, while the Msx1-binding site (24) contains the TAAT core sequence recognized by most homeoproteins. The nucleotide sequences of the Nkx2.1 (CAAGTG) and the Msx1 (TAATTG) binding sites are similar to the NKX3.1 consensus sequence, but differ at nucleotides proposed to be important for optimal binding (1,25). The results, shown in Figure 3A, suggest that wild-type NKX3.1 will bind to the Nkx2.1 and Msx1 consensus sites. These results are in agreement with a previous report that showed that a murine Nkx3.1 homeodomain polypeptide bound both CAAGTG and TAATTG sequences (5). However, the present data indicate that the NKX3.1 TAAGTA consensus site was reproducibly a slightly stronger competitor for NKX3.1 binding than either the CAAGTG or TAATTG binding sites (Fig. 3B). Using increasing amounts of protein with a constant probe concentration, Chen *et al.* also observed only minor differences in the binding affinity of Nkx2.5 when the CAAGTG NK-2 consensus sequence was substituted with either TAATTA or TAAGTG (15). As a control for non-specific competitor binding, an Oct-1 CTAAAC binding site, which was not bound by NKX3.1 (Fig. 1), was included as a competitor at 500 nM, the highest concentration used in the binding assay. There was no apparent decrease in the band representing protein-bound DNA in the presence of CTAAAC.

#### Effect of mutations in the TAAGTA consensus site on NKX3.1 binding

NKX3.1 belongs to the NK class of homeoproteins, but preferentially bound the novel TAAGTA site rather than the Nkx2.1 CAAGTG site. Competitive gel shift experiments were used to isolate the nucleotide(s) important for preferential binding of NKX3.1 to the TAAGTA site. Sequences were generated to replace either T with C at position 1 (TAAGTA→CAAGTA)

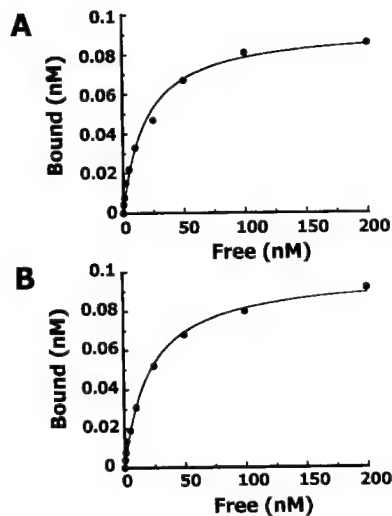


**Figure 3.** Wild-type NKX3.1 preferentially binds the NKX3.1 consensus sequence rather than Nkx2.1 and Msx1 binding sites. (A) Binding affinity of NKX3.1 to TAAGTA was compared with NKX3.1 binding to the Nkx2.1 and Msx1 binding sites by a competitive gel shift assay. Wild-type NKX3.1 was incubated with a probe containing the NKX3.1 consensus sequence in the absence or presence of unlabeled competitor DNA. Competitors were included at concentrations of 50, 250 and 500 nM. Free probe and protein-bound complex are indicated with arrows. (B) The data were quantitated and normalized to wild-type NKX3.1 binding to probe with no competitor. Nucleotide sequences of the competitors are listed at the bottom of the graph. closed circles, TAAGTA; closed squares, CAAGTG; closed triangles, TAATTG.



**Figure 4.** Effect of mutations in the TAAGTA consensus site on NKX3.1 binding. The effects of mutations in the NKX3.1 consensus site were analyzed by competitive gel shift assay. Wild-type NKX3.1 was incubated with a probe containing the NKX3.1 consensus sequence in the absence or presence of unlabeled competitor DNA. Competitors were included at concentrations of 50, 250 and 500 nM. Free probe and protein-bound complex are indicated with arrows. Nucleotide sequences of the competitors are listed at the bottom of the figure.

or A with G at position 6 (TAAGTA→TAAGTG) of the NKX3.1 consensus binding site. The results of the competition assay (Fig. 4) indicate that replacing A at position 6 of the consensus site with G did not affect binding competition relative to the native TAAGTA site. However, replacing T with C at position 1 decreased binding competition, suggesting

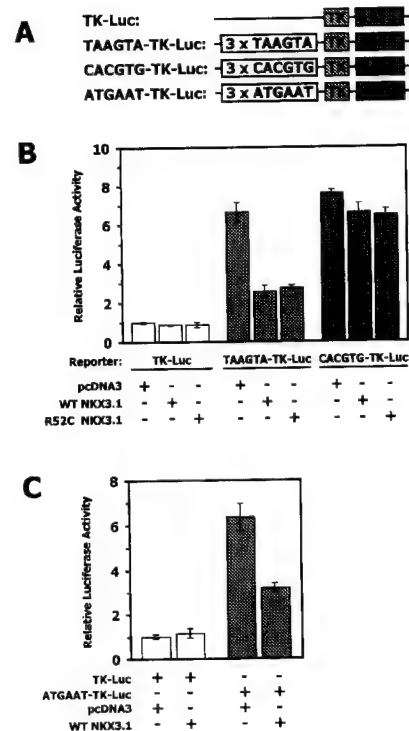


**Figure 5.** Wild-type and R52C NKX3.1 exhibit similar DNA binding affinities. The equilibrium dissociation constants for (A) wild-type and (B) R52C NKX3.1 binding to an NKX3.1 consensus site were determined by gel mobility shift using a constant amount of radiolabeled probe ( $1 \times 10^{-10}$  M) with various protein concentrations ( $0.5$ – $200 \times 10^{-9}$  M). A plot of the quantitated data is shown as bound protein–DNA complex concentration as a function of free protein concentration. The data were analyzed by non-linear least squares as described in Materials and Methods.

that T instead of C at the first position of the consensus sequence is preferred for optimal NKX3.1 binding. Mutating the TAAGTA site to either TAAGCC (Mut3) or TAAGAT (Mut4) significantly decreased competitor binding (Fig. 4), indicating that the 3'-dinucleotide of the NKX3.1 consensus site is important for protein binding. These results are in agreement with previous findings by Damante *et al.* (25,26) indicating that the 3'-dinucleotide of the Nkx2.1 site (CAAGTG) is necessary for optimal protein binding. Sequences selected by wild-type and R52C NKX3.1 in the SAAB assay (Fig. 1) exhibited a high frequency of A/T-rich nucleotides flanking the TAAGTA binding site. To investigate the importance of the A/T flanking sequence for wild-type NKX3.1 binding, a competitive gel shift assay was performed using NKX3.1 binding sites with A/T flanking nucleotides replaced with G/C either upstream (Mut5) or downstream (Mut6) of the TAAGTA sequence (Fig. 4). Both Mut5 and Mut6 had similar competitor efficacy as the native NKX3.1 binding sequence, indicating that the predominance of A/T nucleotides flanking the TAAGTA site did not significantly influence NKX3.1 DNA binding. It is possible that the high frequency of A/T base pairs flanking the NKX3.1 consensus site was an artifact of the binding assay. In fact, previous selection assays using NK class homeoproteins also yielded sequences with a relatively high A/T content outside their respective consensus binding sites (15,17).

#### Wild-type and R52C NKX3.1 exhibit similar binding affinities

To determine if the polymorphism at position 52 affected DNA binding affinity, the dissociation constants for wild-type and



**Figure 6.** NKX3.1 represses reporter gene transcription. (A) Diagram representing reporter plasmids used to analyze the effect of NKX3.1 on reporter gene transcription. (B) Reporter plasmids were used to transiently transfect TSU-Pr1 cells co-transfected with wild-type or R52C NKX3.1 expression plasmids or empty expression plasmid (pcDNA3) as described in Materials and Methods. CMV-*Renilla* plasmid was included as a control for transfection efficiency. (C) TSU-Pr1 cells were co-transfected with expression plasmid and empty reporter vector or reporter vector containing NKX3.1 binding sites in the antisense direction. CMV-*Renilla* plasmid was included as a control for transfection efficiency.

R52C NKX3.1 binding to the TAAGTA consensus site were measured. Increasing amounts of protein were incubated with a constant amount of radiolabeled probe. Following quantitative analysis, the data were plotted and analyzed by non-linear least squares for dissociation constant determination (Fig. 5). The dissociation constants for the proteins were nearly identical, exhibiting  $K_d$  values of 20 nM for wild-type and 22 nM for R52C NKX3.1. The results indicate that mutation at position 52 of NKX3.1, which lies N-terminally outside the homeo-domain region, does not affect DNA binding affinity.

#### NKX3.1 acts as a transcriptional repressor

Luciferase reporter vectors (Fig. 6A) were constructed with three tandem NKX3.1-binding sites in the sense (TAAGTA-TK-Luc) and antisense (ATGAAT-TK-Luc) directions upstream from a herpes simplex virus TK promoter. Co-transfection of TSU-Pr1 cells with TAAGTA-TK-Luc and an NKX3.1 expression vector resulted in decreased transcription compared with control cells co-transfected with TAAGTA-TK-Luc and empty expression vector (Fig. 6B), suggesting that in this context NKX3.1 behaved as a transcriptional

repressor. There was no apparent difference in the relative luciferase activities of wild-type and R52C NKX3.1 co-transfected with TAAGTA-TK-Luc, suggesting that the polymorphism did not affect NKX3.1 transcriptional repression *in vitro*. Previous binding studies have shown that a murine Nkx3.1 homeodomain will not bind to a CACGTG sequence (5). When the NKX3.1-binding sites were mutated to CACGTG, the relative luciferase activity using a NKX3.1 expression plasmid had little effect on transcription, indicating that transcriptional repression with reporter constructs containing three copies of TAAGTA was due to specific binding of NKX3.1. The level of transcriptional repression using ATGAAT-TK-Luc and wild-type NKX3.1 expression vector was similar to that observed using TAAGTA-TK-Luc and wild-type expression vector (Fig. 6C), indicating that the orientation of the TAAGTA sequence does not affect transcriptional repression due to NKX3.1 binding. Insertion of the TAAGTA and CACGTG inserts increased luciferase activity relative to the unmodified TK-Luc reporter plasmid. Based on these results, it is likely that these inserts contain binding sites for transcriptional activators endogenously expressed in TSU-Pr1 cells. For example, the TAAGTA insert contains multiple ATTA sequences (the complement of TAAT), which is bound by most homeoproteins (1). Also, the CACGTG sequence is a high affinity MYC-binding site (27).

## DISCUSSION

Using purified fusion proteins in a random DNA selection assay, a TAAGTA consensus binding sequence for wild-type and R52C NKX3.1 was identified. The TAAGTA consensus site has not been isolated for any other NK class homeoprotein. Previously reported DNA binding sites for NK class homeoproteins include CAAGTG for Nkx2.1, AAAGTG for *Caenorhabditis elegans* ceh-22 and NAAGTG for Nkx2.5 (14,15,28). Structural and mutational studies indicate that amino acid residues of helix III and the N-terminal arm of the homeodomain primarily determine DNA binding specificity (26). Amino acid residues 6–8 of the N-terminal arms of the NK-2 and Nkx2.1 homeodomains have been implicated as the primary determinants of the preferred nucleotide for binding at the 5'-end of the 5'-CAAG-3' core binding site (26,29). The homeodomains of NK-2 and Nkx2.1 have Val, Leu and Phe at positions 6–8, while NKX3.1 has Ala, Ala and Phe at these positions. The variation in side-chain volume of residues 6 and 7 may result in preferential binding by NKX3.1 of T instead of C at the first position of the TAAGTA binding site. When Leu at position 7 of the NK-2 homeodomain was mutated to Ala, the binding affinity for the CAAGTG site decreased by one order of magnitude (29), further suggesting that side-chain volume of residues 6 and 7 is an important determinant of homeodomain binding specificity. Attempts to alter binding preference from TAAGTA to CAAGTA by mutation of Ala to Leu at position 7 of the NKX3.1 homeodomain were unsuccessful (our unpublished data). Therefore, it is likely that a relatively small side-chain volume at position 6, in addition to position 7, of the NKX3.1 homeodomain is required for preferential binding of T instead of C in the TAAGTA sequence. NK class homeodomains characteristically have Tyr at position 54 (2), which confers preference for atypical DNA binding sites

relative to the canonical TAAT binding site recognized by most homeodomains (26). The tyrosine residue at position 54 of the homeodomain is reportedly involved in the recognition and preferential binding of guanosine at the 3'-end of the 5'-CAAG-3' core binding site for Nkx2.1 (26). NKX3.1 also has Tyr at position 54 of the homeodomain, therefore, it is not surprising that wild-type NKX3.1 preferentially binds a TAAG rather than a TAAT core sequence (Fig. 3). Optimal DNA binding of Nkx2.1 requires a TG dinucleotide following the CAAG core binding site (25). The consensus DNA binding sequence for NKX3.1 exhibited a TA dinucleotide following the TAAG core. Structural studies of Nkx2.1 have revealed that Gln at position 50 of the homeodomain region contacts the TG dinucleotide flanking the 3'-end of the 5'-CAAG-3' core binding sequence. This residue is conserved among NK-2 and NK-3 homeodomains (2). Therefore, based on sequence homology, it is predicted that NKX3.1 would also preferentially bind a TG dinucleotide instead of TA following the TAAG core binding site. Although our binding site selection data suggest that NKX3.1 prefers a TA dinucleotide following the TAAG core (Fig. 4), competitive binding experiments suggested that replacing the A with G in the dinucleotide (TAAGTA→TAAGTG) had no observable effect on wild-type NKX3.1 binding (Fig. 4), providing evidence that the nucleotides may be interchangeable. In fact, a previous report describing the DNA sequences within the thyroglobulin promoter important for Nkx2.1 transactivation identified a CAAGTA binding site as well as CAAGTG (30).

Based on the results of competition gel shift assays, NKX3.1 displays a high degree of binding promiscuity (Fig. 3). It is likely that site-specific DNA binding of NKX3.1 *in vivo* is enhanced by post-translational modification and/or protein-protein interaction. For example, phosphorylation and intermolecular disulfide bond formation have been shown to increase DNA binding affinities of Nkx2.1 and Nkx2.5 (31–33). Also, protein interaction of NK-3 with the homeodomain-interacting protein kinase 2 (HIPK2) significantly increased NK-3 DNA binding affinity and transcriptional repression, independently of NK-3 phosphorylation by HIPK2 (34). In addition, Choi *et al.* have recently shown that the co-repressor protein Groucho, which by itself does not repress transcription, interacts with the homeodomain region of NK-3 to increase *in vitro* transcriptional repression (35).

There were no apparent differences in DNA binding or transcriptional repression between wild-type and R52C NKX3.1 under the described conditions. The polymorphism occurs at position 52 of NKX3.1, which is not located within the homeodomain. It has been suggested that regions outside the homeodomain may not contribute to DNA binding (25). However, it is possible that wild-type and R52C NKX3.1 differ functionally *in vivo* due to post-translational modification(s). For example, our preliminary data suggest that the polymorphism at position 52, which lies within a putative phosphorylation site at Ser48 (unpublished observation), may affect DNA binding through altered levels of phosphorylation (manuscript in preparation). Also, introduction of a cysteine at position 52 may affect redox sensitivity of DNA binding by the formation of aberrant inter- or intramolecular disulfide bonds. Further analysis may provide insights into the effects of the polymorphism on the function of NKX3.1.

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# Loss of NKX3.1 Expression in Human Prostate Cancers Correlates with Tumor Progression<sup>1,2</sup>

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## ABSTRACT

*NKX3.1* is a prostate-specific homeobox gene located on chromosome 8p21. In the mouse, *Nkx3.1* has growth-suppressive and differentiating effects on prostatic epithelium. Mutations of the coding region of *NKX3.1* were not found in human prostate cancer, failing to support the notion that *NKX3.1* was a tumor suppressor gene. To study the expression of *NKX3.1* protein in human tissues and prostate cancer, we derived a rabbit antiserum against purified recombinant *NKX3.1*. Among normal human tissues, *NKX3.1* expression was seen in testis, in rare pulmonary mucous glands, and in isolated regions of transitional epithelium of the ureter. *NKX3.1* was uniformly expressed in nuclei of normal prostate epithelial cells in 61 histological sections from radical prostatectomy specimens. We analyzed 507 samples of neoplastic prostate epithelium, most of which were contained on a tissue microarray that contained samples from different stages of prostatic neoplasia. We observed complete loss of *NKX3.1* expression in 5% of benign prostatic hyperplasias, 20% of high-grade prostatic intraepithelial neoplasias, 6% of T1a/b samples, 22% of T3/4 samples, 34% of hormone-refractory prostate cancers, and 78% of metastases. Our data show that *NKX3.1* expression is highly, but not exclusively, specific for the prostate. Loss of *NKX3.1* expression is strongly associated with hormone-refractory disease and advanced tumor stage in prostate cancer ( $P < 0.0001$ ).

## INTRODUCTION

*NKX3.1* is a homeobox gene with prostate-specific expression in the adult (1). *NKX3.1* maps to chromosome 8p21, a region that undergoes LOH<sup>4</sup> in ~75% of prostate cancer specimens (2-6). For this reason, *NKX3.1* was a candidate target gene for disruption by the 8p21 LOH. However, mutational analysis failed to find any tumor-

specific mutations of *NKX3.1* in human prostate cancer tissues (2). *NKX3.1* has potent growth-suppressing and differentiating effects on prostatic epithelium. Mice heterozygous for targeted disruption of *Nkx3.1* have abnormal prostate morphology with overgrown and dysplastic epithelium (7). Disruption of prostate epithelial morphology and dysplasia is more severe in *Nkx3.1*-null mice (7). The suggestion that gene dosage, and therefore the amount of protein, may be important for the growth-suppressor effects of *NKX3.1* prompted us to study its expression in human prostate cancer specimens.

This report describes the derivation of an antiserum against purified recombinant *NKX3.1* protein and the immunohistochemical expression of *NKX3.1* in normal human tissues and in prostate cancer specimens. One report of *NKX3.1* mRNA expression in human prostate cancer tissues described increased expression in prostate cancers compared with adjacent normal tissue (8). Our data examining *NKX3.1* protein expression support the opposite conclusion. We demonstrate that loss of the expression of this growth suppressor correlates with prostate tumor progression.

## MATERIALS AND METHODS

**Expression and Purification of *NKX3.1* Recombinant Protein.** A 3'-truncated cDNA of wild-type *NKX3.1*, including nucleotides 1-581 and excluding the region that coded for the COOH-terminal region of the protein downstream from the homeodomain, was inserted into pMAL-C2g vector (New England Biolabs, Waltham, MA) at the *SalI* and *EcoRI* restriction sites. Fusion plasmid was transformed into BL-21-competent cells (Stratagene, La Jolla, CA). An overnight culture of bacteria containing the fusion plasmid was induced with 0.5 mM IPTG for 2 h. MBP<sup>9</sup>-NKX fusion protein was purified by affinity chromatography with amylose resin (New England Biolabs). Purified fusion protein was cleaved with 0.05 µg of genease I (New England Biolabs) per 10 µg of fusion protein at room temperature for 24 h. Pure recombinant *NKX3.1* was purified again by DEAE ion exchange chromatography.

**Production of *NKX3.1* Polyclonal Antibody.** Twenty-five µg of purified *NKX3.1* recombinant protein in TiterMax adjuvant emulsion (CytRx Corporation, Norcross, GA) were inoculated into New Zealand White rabbits. The total volume of the initial inoculation was 400 µl, and a 200-µl boost was administered 3-4 weeks later. Rabbits were test bled 3-4 weeks after the initial inoculation and after the second boost. Rabbit anti-NKX3.1 antibody was purified by affinity chromatography by successive passes through BL-21-MBP CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala Sweden) followed *NKX3.1* CNBr-activated Sepharose 4B.

**Immunohistochemical Staining.** Deparaffinized tissue sections were preheated in 10 mM sodium citrate solution for 20 min in a Black and Decker vegetable steamer. NKX3.1 antibody diluted 1:1000 in blocking buffer (1:70 dilution of goat serum in PBS) was incubated on slides for 1 h at room temperature. Sections were then incubated with 1:200 diluted biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min and ABC solution (Vector Labs) for another 30 min. VIP peroxidase substrate (Vector Labs) was used to stain tissues, which were then counterstained with methyl green.

**Tissues.** Sections of normal human tissues were obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource. Prostate tissue specimens for normal tissue and the 30 prostate cancers in the validation set came from the Lombardi Cancer Center Histopathology and Tumor Core Facility. These specimens were collected at the time of RP and therefore represent specimens from clinical stage I and II prostate cancers. All histological diagnoses were confirmed

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<sup>2</sup> The authors of this report made the following contributions: C. B. isolated and purified the recombinant protein, made the antibody, developed the immunohistochemical technique, and stained the histologic sections. L. B. helped develop the tissue microarray technology and constructed the prostate cancer tissue microarray. H. J. V. made the expression vectors for the recombinant fusion protein. R. S. performed the statistical analysis. E. E. L. did the Gleason scoring of histologic samples. N. W. supported tissue microarray construction and histologic review of specimens. G. S. contributed to the development of tissue microarray technology and coordinated the tissue collection and the microarray facility in Basel. T. C. G. helped to select the specimens for the array and provided important clinical information. P. K. collected specimens of hormone-refractory prostate cancer and supporting clinical information. J. K. and O-P. K. developed the tissue microarray technology for high-density arraying of the clinical prostate specimens. E. P. G. directed the research on *NKX3.1*, performed the review of *NKX3.1* staining and scoring, analyzed the data, and prepared the figures.

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<sup>4</sup> The abbreviations used are: LOH, loss of heterozygosity; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MBP, maltose-binding protein; RP, radical prostatectomy; BPH, benign prostatic hyperplasia; PIN, prostatic intraepithelial neoplasia; HR, hormone-refractory.

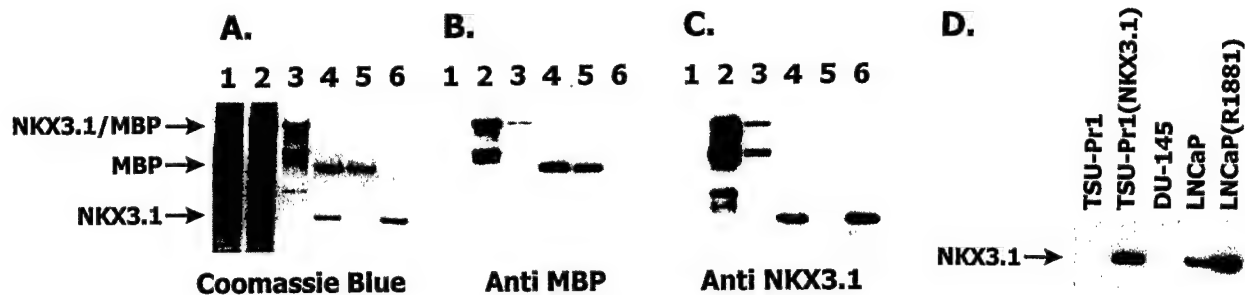


Fig. 1. Antibody to recombinant NKX3.1. A, Coomassie blue staining of samples from purification of recombinant protein. Lane 1, total *E. coli* protein extract prior to IPTG induction; Lane 2, total *E. coli* protein extract after IPTG induction; Lane 3, NKX3.1/MBP fusion protein from amylose column; Lane 4, DEAE-Sepharose-purified fusion protein cleaved with genenase I; Lane 5, DEAE-Sepharose-purified MBP after genenase I digestion of fusion protein; Lane 6, DEAE-Sepharose-purified NKX3.1 after genenase I digestion of fusion protein. B, Western blot of gel as in A, using anti-MBP. C, Western blot of gel as in A, using purified rabbit antibody to NKX3.1. D, Western blot with anti-NKX3.1. Contents of the lanes are labeled.

by staining parallel sections with H&E. Specimens were reviewed by one of us (E. L.) for purposes of assigning Gleason grades.

**Tissue Microarrays.** The prostate tissue microarray was constructed as described previously (9). Briefly, core tissue biopsies (diameter, 0.6 mm) were taken from paraffin-embedded prostate tumors (donor blocks) and precisely arrayed into a new recipient paraffin block (35 × 20 mm) with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). After the array block was constructed, multiple 4-μm sections were cut with a microtome using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ). Formalin-fixed and paraffin-embedded tumor and benign control specimens were obtained from the archives of the Institutes for Pathology, University of Basel (Basel, Switzerland), the Cantonal Institute for Pathology (Liestal, Switzerland), and the Tampere University Hospital (Tampere, Finland). The tissue microarray initially contained 632 specimens from all stages of tumor progression. The presence of tissue conforming to the histopathological category assigned in the original assembly was verified by review of an H&E-stained section within 50 μm of the section stained for NKX3.1; this review identified 477 tissue core specimens that were included in the analysis. Tissue samples included BPH as control ( $n = 43$ ); primary tumors with stage T1<sub>a/b</sub> according to International Union Against Cancer criteria (10), incidentally discovered after transurethral resection for presumed BPH ( $n = 109$ ); clinically localized tumors obtained from RP specimens (clinical stage T2;  $n = 110$ ); primary, locally advanced tumors (clinical stage T3/4) treated by transurethral resection ( $n = 27$ ); distant metastases collected from autopsies of patients who had died from end-stage metastatic prostate cancer ( $n = 35$ ); and 108 local recurrences after hormonal therapy failure, including 65 transurethral resections from living patients and 43 specimens obtained from autopsies. Tumor grading on the original tissue sections was performed according to Gleason (11). The array also included 54 cores from high grade PIN lesions; however, because of the focal nature of PIN, we verified the H&E staining of each sample on the array and identified only 20 as clearly showing high-grade PIN in the tissue core specimens on the array.

**Statistical Methods.** Specimens were available from 30 radical prostatectomies. These specimens were assessed for Gleason score and NKX3.1 expression to determine whether NKX3.1 expression differed among specimens with at least one Gleason grade  $\geq 4$  compared with those with both grades  $< 4$ . The prostate tissue samples available for tissue microarray analysis were ordered by increasing disease severity for the following classifications: BPH, PIN, T1 tumors, RP specimens, and T3/4 tumors. Specimens available from HR samples and metastatic disease represented more severe disease than the previously mentioned tissues, but their position in severity status relative to each other was unknown. Of primary interest was whether there is a decrease in NKX3.1 expression with increasing disease status. Two separate questions were addressed. The first was whether a trend in NKX3.1 expression is present with disease status BPH through T3/4 in the order listed above, with HR tumors as the most severe disease status. The second question was similar, except that metastatic disease rather than HR tumors was the most severe disease. Of additional interest was whether the combined group of T1<sub>a/b</sub> and RP tissues differed from T3/4 and whether it differed from metastatic tumors. These questions were tested using a Jonkheere-Terpstra test as implemented in StatXact (Cytel). Unless specified below, all tests were considered significant

if  $P < 0.05$ . To control for the two tests using HR or metastatic tumors as the sixth tissue type, the decrease in NKX3.1 expression was considered significant if the two-sided  $P$  was  $< 0.025$ . Specific pairwise comparisons with BPH through T3/4 were performed for HR or metastatic tumors provided the overall test was significant. Similarly, the two comparisons of T1<sub>a/b</sub> with either T3/4 or metastatic tissue were considered significant for  $P < 0.025$ .

## RESULTS

Recombinant NKX3.1 was made as an MBP fusion protein in *Escherichia coli*. Cleavage of the fusion protein with genenase yielded electrophoretically pure NKX3.1 (Fig. 1A). Antibody to MBP did not react with cleaved NKX3.1, indicating complete cleavage of NKX3.1 from the fusion protein (Fig. 1B). Rabbit antiserum derived against purified recombinant NKX3.1 reacted only with NKX3.1 on Western blot and not with either *E. coli* proteins or MBP (Fig. 1C). The antiserum recognized 32-kDa NKX3.1 in TSU-Pr1 cells transfected with an NKX3.1 expression plasmid, but detected no proteins in TSU-Pr1 cells because they express  $< 1/100$  the level of NKX3.1 mRNA found in LNCaP cells (Fig. 1D).<sup>5</sup> The induction of NKX3.1 mRNA by androgen treatment of LNCaP cells has been described and was reflected in the induction of NKX3.1 protein after R1881 treatment of LNCaP cells (Fig. 1D; Refs. 1, 12).

The expression of NKX3.1 mRNA is restricted in the adult mouse and humans. In the mouse, expression is seen only in the prostatic lobes and the bulbourethral gland (7). Expression in humans was seen predominantly in the prostate, but low levels of mRNA were also detected in testis (1). There also appeared to be signals in peripheral blood lymphocytes (1). In fact, our attempts to use NKX3.1 as a prostate-specific marker failed because reverse transcription-PCR detected transcripts in female peripheral blood.<sup>6</sup> To clarify the expression of NKX3.1 in human tissues, we did immunohistochemical staining of 16 human tissues, including prostate, brain, heart, lung, kidney, testis, bladder, ureter, skin, liver, spleen, bone marrow, small and large intestine, breast, and endometrium, using our rabbit antiserum. On the basis of preliminary studies with LNCaP cells and NKX3.1-transfected TSU-Pr1 cells, we expected to find this homeobox protein localized to the nucleus.

We detected expression of NKX3.1 in testis, confirming the results for mRNA expression (Fig. 2, A and B). We also noted expression of NKX3.1 in rare mucous glands of the lung (Fig. 2, C and D). Lastly, we found expression of NKX3.1 in groups of ureteral epithelial cells periodically spaced along the lumen of the ureter (Fig. 2, E and F). Bladder transitional epithelium contained rare single cells with nu-

<sup>5</sup> H. J. Voeller and E. P. Gelmann, unpublished data.

<sup>6</sup> H. J. Voeller and E. P. Gelmann, unpublished data.



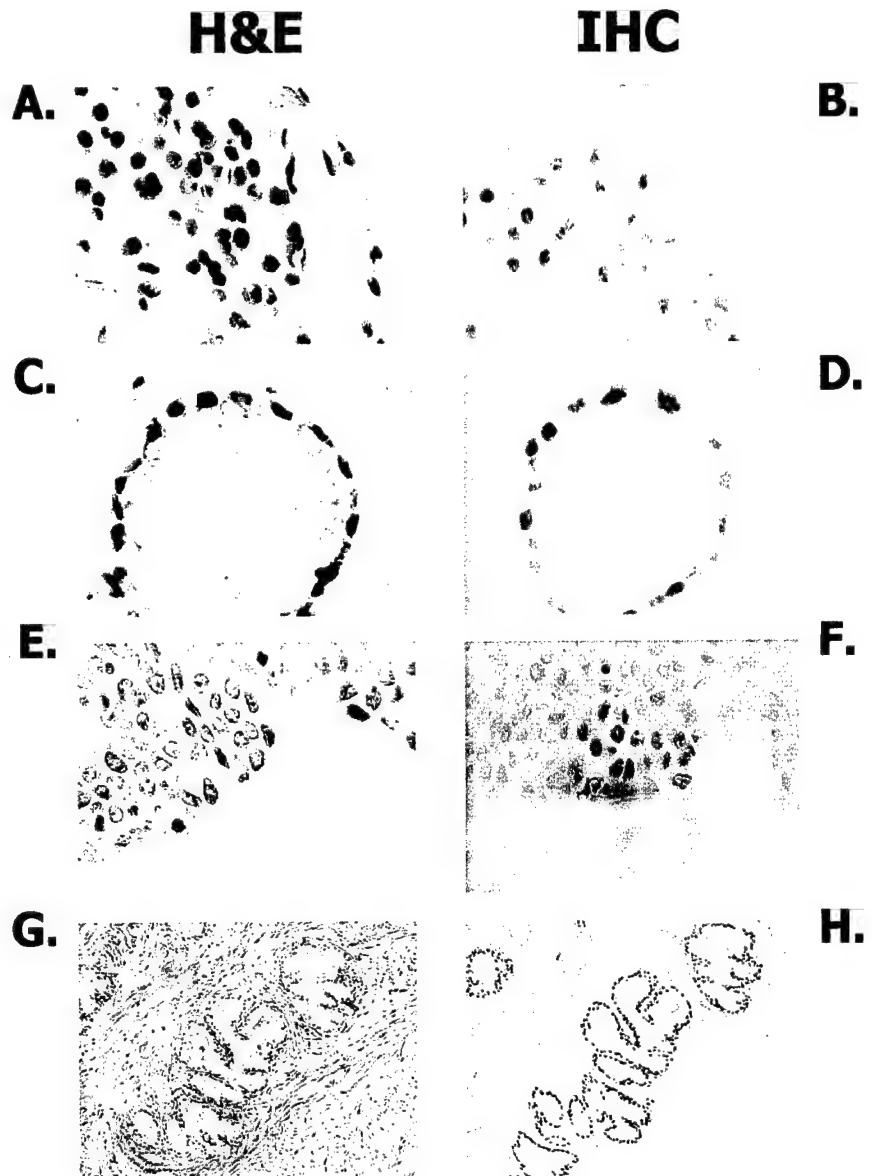


Fig. 2. Immunohistochemical staining of normal human tissues with anti-NKX3.1. Sections were cut in parallel and stained with either H&E (A, C, E, and G) or anti-NKX3.1 (B, D, F, and H). Images A–F were captured digitally using  $\times 400$  microscopic magnification; G and H, were captured digitally using  $\times 63$  magnification. A and B, normal testis seminiferous tubules. C and D, pulmonary mucous gland. E and F, ureteral transitional epithelium. G and H, prostate gland. IHC, immunohistochemistry.

clear staining (not shown). We found no expression in tissues that contained blood cells, including bone marrow and spleen. Nonmalignant prostatic epithelial cells had uniformly positive nuclear staining for NKX3.1. This was seen in 61 RP specimens (Fig. 2, G and H).

To determine the expression of NKX3.1 in neoplastic prostate epithelium, we analyzed a tissue microarray that contained a spectrum of tissue samples providing a cross-section of prostate tumor progression. To validate the data in the array from one category of tissue, we performed conventional immunohistochemical staining on a separate set of 61 embedded tissue blocks from prostatectomy specimens. Thirty of these blocks contained malignant foci that were scored for NKX3.1 expression and compared with the results of the RP samples in the tissue microarray.

Neoplastic prostate epithelium was found to display three different patterns of immunostaining for NKX3.1 expression. Many samples stained uniformly for NKX3.1. Some samples stained heterogeneously, with some malignant cells stained and adjacent cells not stained. Some samples displayed no staining for NKX3.1. In samples in which malignant cells did not express NKX3.1, adjacent normal epithelial cells were invariably positive, providing an internal control for the quality of the specimen. The patterns of staining are shown in Fig. 3. For the purposes

of analyzing the 477 microarray samples and the 30 sections, uniform staining was awarded a score of 2, heterogeneous staining a score of 1, and samples that did not stain were scored 0.

The results of staining for the tissue microarray are shown in Table 1. Whereas the majority of samples from early-stage cancers stained uniformly for NKX3.1 in epithelial cell nuclei, the number of samples with heterogeneous or negative staining increased among the locally advanced T3/4 and HR samples. There was a significant reduction of NKX3.1 expression in advanced prostate cancer as defined by either HR ( $P < 0.0001$ ) or metastatic disease ( $P < 0.0001$ ). HR demonstrated significantly lower NKX3.1 expression than BPH ( $P < 0.0001$ ), T1<sub>a/b</sub> ( $P < 0.0001$ ), and RP ( $P = 0.013$ ). Metastatic samples differed from all tissue samples BPH through T3/4 ( $P < 0.0001$  for each). The combined group of T1<sub>a/b</sub> tumors and RP samples had significantly higher NKX3.1 expression than metastases ( $P < 0.0001$ ) but did not achieve a significant difference compared with T3/4 ( $P = 0.061$ ; Refs. 13, 14).

Seventy-eight percent of metastatic samples had no staining for NKX3.1. The integrity of these autopsy samples was confirmed by staining for Ki-67, which was seen to decorate nearly all of the metastatic samples. Because Ki-67 may be more stable than NKX3.1 under conditions found at autopsy, we also compared the HR speci-

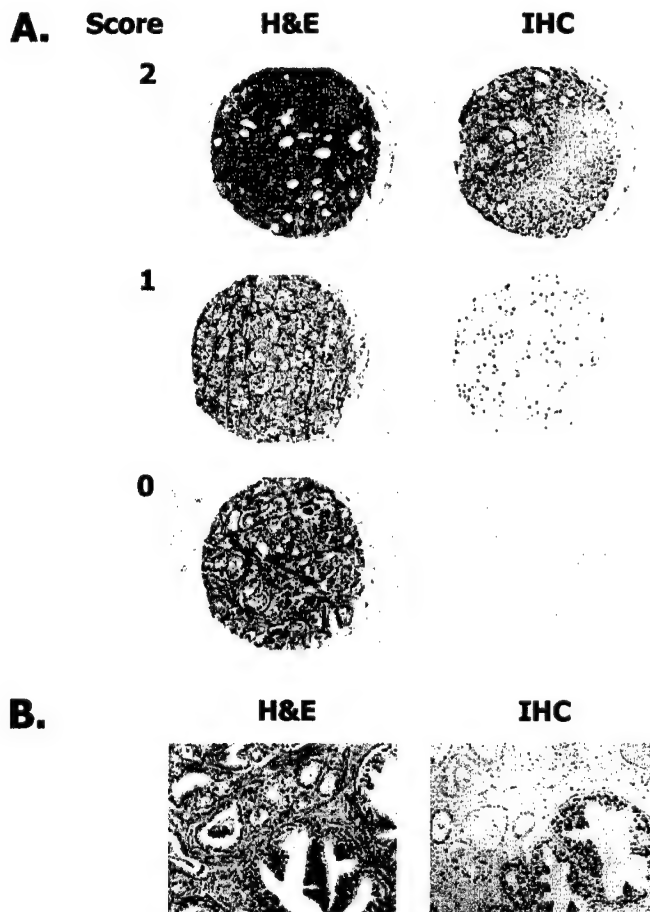


Fig. 3. Immunohistochemical staining of prostatic tissue and tissue microarrays with anti-NKX3.1. A, examples of prostate cancer specimens from tissue microarrays stained with H&E (left) and corresponding section stained with anti-NKX3.1 (right). Staining patterns observed were diffuse staining (2), heterogeneous staining (1), or no staining (0). All tissues were scored according to this scheme, and the data are summarized in Table 1. B, parallel sections from a Gleason score 3 + 3 prostate cancer specimen stained with either H&E (left) or anti-NKX3.1 (right). Images were captured at  $\times 100$ . NKX3.1 expression was absent in the adenocarcinoma (upper left of slide) and present in hyper-trophic glands (lower right of slide). IHC, immunohistochemistry.

mens that were obtained surgically with those obtained at autopsy. Among 128 HR specimens, 43 were autopsy specimens and 85 were surgical specimens. Of the HR autopsy specimens, 23 of 53 (43%) had staining scores of 0 compared with 24 of 85 (25%) surgical HR specimens. Therefore, it is probable that diminished staining of autopsy-derived material resulted, in part, from antigen degradation.

However, staining of HR autopsy specimens scored positive twice as often as staining of autopsy-derived metastases.

The series of 30 RP specimens analyzed as conventional sections cut from paraffin blocks gave nearly identical results to the distribution of staining patterns found among the RP specimens in the tissue microarray. The microarray RP samples gave a nearly identical distribution of staining scores compared to these 30 paraffin block samples; therefore, the staining of conventional samples validated the tissue microarray data and confirmed that a fraction of early-stage prostate cancers lose expression of NKX3.1.

The 30 samples from paraffin blocks were subjected to histological grading (11). In contrast to the relationship between loss of NKX3.1 staining and prostate tumor progression, we found no relationship between NKX3.1 staining scores and Gleason scores in the 30 RP blocks. The distribution of NKX3.1 staining results over the range of tumor grades as measured by Gleason score is shown in Table 2. Samples were compared across all scores and compared as groups with at least one grade  $\geq 4$  versus both grades  $< 4$ . There was no evidence indicating that patients with lower NKX3.1 expression had higher Gleason scores ( $P = 0.611$ ; Ref. 13).

## DISCUSSION

Our data show that NKX3.1 expression is lost in a significant fraction of early-stage prostate cancer and that loss of expression correlates with tumor progression. Because NKX3.1 has differentiating and growth-suppressing effects in the mouse prostate, it is tempting to speculate that NKX3.1 plays a tumor-suppressor function in human prostate cancer. If, similar to the mouse, the human prostatic epithelium is sensitive to the level of NKX3.1 protein, then diminution in the level of NKX3.1 expression, even in tissues that demonstrate heterogeneous staining, may play a role in the pathogenesis or progression of human prostate cancer. NKX3.1 staining was found in three patterns in the tissue samples. Most samples, except for metastases, showed diffuse staining of both normal and neoplastic prostatic epithelial cells. In our experience, all normal prostate epithelial cells express NKX3.1. NKX3.1 expression decreases with disease severity. That fact combined with the lack of relationship between NKX3.1 expression and the Gleason score means that NKX3.1 expression has the potential to be a promising new prognostic marker if it is associated with patient survival. Further studies are warranted in this area. A previous report that *NKX3.1* mRNA expression was increased in prostate cancer tissues compared with adjacent normal tissues arrived at a different conclusion from our findings (8). If the results of Xu *et al.* (8) are confirmed, it would lead to the conclusion that control of NKX3.1 expression occurs at the posttranscriptional level.

In the survey of 61 tissue sections, we found no example of

Table 1. Frequencies of progression array staining for NKX3.1

NKX3.1 staining score	Tissue microarray samples, n (%)							RP sections, <sup>a</sup> n (%)
	BPH	PIN	T1 <sub>a/b</sub>	RP <sup>a</sup>	T3/4	HR	Meta <sup>b</sup>	
2	36 (84)	9 (45)	83 (76)	68 (62)	14 (52)	64 (50)	4 (10)	19 (63)
1	5 (12)	7 (35)	19 (17)	24 (22)	7 (26)	20 (16)	5 (13)	7 (23)
0	2 (5)	4 (20)	7 (6)	18 (16)	6 (22)	44 (34)	31 (78)	4 (13)
Total (100%) tissue	43	20	109	110	27	128	40	30
<i>P</i> vs.								
HR	<0.0001 <sup>c</sup>	0.789	<0.0001 <sup>c</sup>	0.013 <sup>c</sup>	0.529			
Meta	<0.0001 <sup>c</sup>	<0.0001 <sup>c</sup>	<0.0001 <sup>c</sup>	<0.0001 <sup>c</sup>	<0.0001 <sup>c</sup>			
T1 <sub>a/b</sub> + RP					0.061		<0.0001 <sup>d</sup>	

<sup>a</sup> RP samples from tissue microarray and paraffin sections presented similar NKX3.1 score profiles.

<sup>b</sup> Meta, metastatic disease.

<sup>c</sup> This test is significant at 0.05. No adjustment was made because these analyses were controlled by the overall test.

<sup>d</sup> This test is significant at the specified  $P < 0.025$ .

Table 2 *NKX3.1 staining and Gleason scores*

NKX3.1 staining score	Gleason score					
	2 + 2	2 + 3	3 + 3	3 + 4	4 + 4	4 + 5
2	1	3	7	6	2	0
1	1	3	2	0	1	0
0	1	0	1	0	1	1

nonmalignant prostatic glands failing to stain for NKX3.1. In the array, two BPH specimens displayed no staining. We cannot state at this time whether this represents a background of tissues that failed to stain for technical reasons or a subset of prostatic hyperplasia with true loss of NKX3.1 expression. Analysis of PIN samples revealed that more than half had reduced or absent NKX3.1 expression. Therefore, NKX3.1 may also play a role in the development of prostate cancer. A larger number of PIN lesions need to be analyzed to elucidate the role of NKX3.1 in prostate cancer development.

The finding that NKX3.1 expression was lost most often in metastases is consistent with the notion that metastatic disease is the most dedifferentiated state of prostate cancer. It may also be that NKX3.1 expression is under the control of prostate stromal cells. In murine tissue recombinants of neonatal epithelium and mesenchyme from the urogenital sinus, only tissues that underwent prostatic differentiation expressed *Nkx3.1* (7). On the other hand, the quality of the specimens, particularly from autopsies, could have resulted in sample degradation and diminished ability to detect NKX3.1. The mechanism of modulating NKX3.1 expression in human prostate cancer remains to be elucidated. It has not been determined whether NKX3.1 undergoes LOH in those tissues that display LOH at 8p21. Therefore, it is possible that loss of a single NKX3.1 allele as a result of LOH at 8p21 could down-regulate NKX3.1 expression. Because NKX3.1 is a differentiating protein, its expression may be regulated by gene methylation. We presently are characterizing the upstream sequences of NKX3.1 to identify regions that may be targets for gene silencing by methylation. Methylation is an important mechanism for loss of differentiated functions in human cancers such as diminished estrogen-receptor expression in breast cancer (15, 16). It remains to be determined whether promoter methylation plays a role in the down-regulation of NKX3.1 expression in prostate cancer.

The survey of NKX3.1 expression in normal tissues underscores the high degree of prostate specificity in the expression pattern of this protein. The role of NKX3.1 in the function of extraprostatic cells where it was found, bronchial mucous glands, testis, and ureter, is unknown. *Nkx3.1* is not expressed in murine testis, and the *Nkx3.1* (-/-) mice were fertile. There was no obvious ureteral or pulmonary pathology attributed to loss of *Nkx3.1* in the gene-deleted mice (7). If the only apparent action of NKX3.1 is as a prostate-specific repressor, the gene may have application in prostate-specific gene therapy. The potential for application of gene therapy to the treatment of prostate cancer is under active investigation. There may be advantages to the use of suppressor genes with limited tissue-specific effects to minimize toxicity of gene therapy to other organs. Whether ectopic expression of NKX3.1 in organs other than the prostate will have any functional ramifications remains to be shown. In addition, because of its tissue-specific expression in the adult, the NKX3.1 promoter is a potentially useful determinant for prostate-specific expression of exogenous genes. The probasin promoter has been quite useful in generating a murine prostate cancer model by driving organ-specific expression of the SV40 T antigen (17-19). Whether the *Nkx3.1* promoter will have similar effects remains to be shown. Early in murine development, *Nkx3.1* expression occurs in many regions of the embryo and may play a noncritical role in the development of other organs (20, 21). Lastly, the NKX3.1 promoter may have applications

in tissue-specific gene therapy of prostatic disease. The expression of NKX3.1 in other tissues shown in this report will help to identify potential organs for side effects of treatments targeted to the prostate by the NKX3.1 promoter.

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# Occurrence of *NKX3.1* C154T Polymorphism in Men with and without Prostate Cancer and Studies of Its Effect on Protein Function<sup>1</sup>

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## ABSTRACT

*NKX3.1*, a member of the NK class of homeodomain proteins, is expressed primarily in the adult prostate and has growth suppression and differentiating effects in prostate epithelial cells. A C→T polymorphism at nucleotide 154 (*NKX3.1* C154T) is present in ~11% of healthy men with equal distribution among whites and blacks. In a cohort of 1253 prostate cancer patients and age-matched controls, the presence of the polymorphism was associated with a 1.8-fold risk of having stage C or D prostate cancer or Gleason score ≥7 (confidence interval, 1.01–3.22). The *NKX3.1* C154T polymorphism codes for a variant protein that contains an arginine-to-cysteine substitution at amino acid 52 (R52C) adjacent to a protein kinase C phosphorylation site at serine 48. Substitution of cysteine for arginine 52 or of alanine for serine 48 (S48A) reduced phosphorylation at serine 48 *in vitro* and *in vivo*. Phosphorylation of wild-type *NKX3.1*, but not of *NKX3.1* R52C or *NKX3.1* S48A, diminished binding *in vitro* to a high-affinity DNA binding sequence. *NKX3.1* also serves as a transcriptional coactivator of serum response factor. Treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate to phosphorylate *NKX3.1* had no effect on *NKX3.1* coactivation of serum response factor. Neither the R52C nor the S48A substitution affected serum response factor coactivation by *NKX3.1*. We conclude that the polymorphic *NKX3.1* allele codes for a variant protein with altered DNA binding activity that may affect prostate cancer risk.

## INTRODUCTION

Prostate cancer is a neoplasm with a variable natural history that ranges from indolent to aggressive. Low-grade or early-stage disease may have little impact on survival. However, patients with advanced stages or higher histological grades suffer substantial disease-related mortality (1). The occurrence of prostate cancer is influenced to a substantial degree by genetic factors (2, 3). Genetic determinants may affect individual risk for aggressive prostate cancer and, therefore, mortality from prostate cancer. For example, a polymorphic region in the androgen receptor gene affects the incidence of aggressive prostate cancer (4, 5).

*NKX3.1* is an androgen-regulated NK-class homeobox gene with expression in adult mice and humans localized primarily in the prostate (6–9). The *NKX3.1* gene has been conserved during evolution; the murine and human proteins share 63% amino acid identity. The human *NKX3.1* has been mapped to chromosome 8p21 (10), a locus frequently deleted in prostate cancer (11–13). However, no tumor-specific mutations of the *NKX3.1* protein-coding region have been identified by genetic analysis of human prostate cancer samples (10).

Nevertheless, loss of *NKX3.1* expression was found in 6–15% of early-stage prostate cancer, 22% of locally advanced disease, 34% of hormone-refractory localized prostate cancer, and 78% of metastases (14). Decreased expression of *NKX3.1* may have a role in prostate cancer pathogenesis because heterozygous *Nkx3.1* gene-targeted mice displayed a phenotype of prostatic hyperplasia, suggesting that *NKX3.1* haploinsufficiency may be dominant.

In the course of analyzing tumor samples for *NKX3.1* mutations, we found a C→T polymorphism at nucleotide 154 (C154T) that coded for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of *NKX3.1* (10). The polymorphism lay NH<sub>2</sub>-terminal to the homeodomain in a region of the protein that was not conserved between mouse and human. We have determined the frequency of the polymorphism in a population of healthy men and examined its role as a possible risk factor for prostate cancer. We also show that the amino acid change coded by the polymorphism alters *in vitro* and *in vivo* properties of the protein.

## MATERIALS AND METHODS

**Plasmid Construction.** Plasmids expressing full-length wild-type or polymorphic *NKX3.1* fused to maltose-binding protein were generated as described previously (15). A plasmid encoding amino acids 1–184 (nucleotides 1–581) of wild-type *NKX3.1* with an NH<sub>2</sub>-terminal FLAG epitope was constructed. *NKX3.1* point mutants were generated using a Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutant *NKX3.1* cDNAs were fully sequenced to confirm the presence of mutations and to ensure that no additional mutations were introduced.

**Bacterial Expression and Purification of *NKX3.1* Fusion Proteins.** Plasmids expressing wild-type or *NKX3.1* R52C were used to transform competent *Escherichia coli* strain BL21. The proteins were expressed and purified as described previously, using an amylose column that was eluted with 10 mM maltose (New England Biolabs, Beverly, MA; Ref. 15).

***In Vivo* Phosphorylation and Immunoprecipitation.** For labeling of exogenous *NKX3.1*, TSU-Pr1 or LNCaP cells were plated on a 6-cm dish in DMEM containing 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD). At ~90% confluence, cells were transfected with 10 μg of wild-type or polymorphic *NKX3.1* expression vector or empty vector, using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Inc.). The *NKX3.1* constructs contained cDNA for expression of amino acids 1–184, including the NH<sub>2</sub> terminus and homeodomain of *NKX3.1*, because our data and those of others had shown that under the control of a cytomegalovirus promoter, the COOH-terminal truncated protein is expressed at higher levels than the wild-type protein (16).

Forty-eight h post-transfection, cells were labeled with 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub> in carrier-free HCl (Amersham Pharmacia Biotech, Piscataway, NJ) for 3–4 h in phosphate-free DMEM containing 5% dialyzed fetal bovine serum (Life Technologies, Inc.). Labeling of endogenous *NKX3.1* was done in LNCaP cells treated with 10 nM methyltrienolone (R1881; DuPont, Boston, MA). Cells were then treated with 100 nM TPA<sup>3</sup> (Sigma, St. Louis, MO) for 30 min before cell lysis. Labeled *NKX3.1* was immunoprecipitated with either 1.5 μg of

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<sup>3</sup> The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SRF, serum response factor; SMGA, smooth muscle γ-actin; FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2',7'-tetrachlorofluorescein; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine.



anti-NKX3.1 polyclonal antiserum or 20  $\mu$ g of anti-FLAG M2 antibody (Stratagene). Immunoprecipitates were electrophoresed on denaturing 10–20% gradient polyacrylamide gels followed by gel drying and autoradiography for visualization of radiolabeled proteins. Western blot analysis to determine protein levels was performed as described previously (14).

**Phosphoamino Acid Analysis of NKX3.1.** Labeled proteins were excised and eluted from polyacrylamide gels. The eluted protein was digested with 0.15 mg/ml trypsin overnight at 37°C, followed by hydrolysis with 1 ml of 6 N HCl at 105°C for 1 h. The HCl was removed by lyophilization, and the pellet was washed with 1 ml of H<sub>2</sub>O and dried. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis as described previously (17). The identity of *in vivo* phosphorylated amino acids was determined by autoradiography followed by comparison of the autoradiogram with phosphoamino acid standards.

**In Vitro Phosphorylation.** Synthetic peptides (30  $\mu$ g) obtained from Research Genetics, Inc. (Huntsville, AL) or purified fusion proteins (200 ng) were incubated at 30°C for 30 min with 10 ng of a purified protein kinase C  $\alpha$ ,  $\beta$ , and  $\gamma$  isoform mixture (Upstate Biotechnology, Lake Placid, NY) in a buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 5 mM DTT, 0.5 mM CaCl<sub>2</sub>, 100  $\mu$ g/ml phosphatidylserine, 50  $\mu$ M ATP, and 0.11  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP.

**Electrophoretic Mobility Shift Assay.** Gel shift assays were performed as described previously with modifications (15). Double-stranded DNA representing a consensus NKX3.1 binding site had the sequence 5'-GTATATA-AGTAGTTG-3' (15).

**Transcription Assay.** CV-1 fibroblasts were maintained in Modified Improved MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Cells were plated at  $\sim 1\text{--}2 \times 10^5$  cells/well in 12-well plates. Cells were transfected 24 h after plating, using Lipofectamine Plus according to the manufacturer's protocol (Life Technologies, Inc.). Each transfection reaction contained either 0.25  $\mu$ g of SMGA reporter plasmid (a gift from Warren Zimmer, University of South Alabama (Mobile, AL); Ref. 16) or 0.2  $\mu$ g of various NKX3.1 expression plasmids. SRF expression plasmid (0.5  $\mu$ g; a gift from Ron Prywes, Columbia University, New York, NY; Ref. 18) was used as indicated. Total transfected DNA was always kept the same and balanced to 0.5  $\mu$ g with empty vector. Cells were lysed 48 h after transfection, and the lysate was assayed for firefly luciferase activities with Dual Luciferase Reporter Assay Reagents (Promega, Madison, WI).

**TaqMan Assay.** The TaqMan allelic discrimination assay (19) was used to determine the frequency of the polymorphism at nucleotide 154 in prostate DNA samples. Genomic DNA was isolated using the Easy DNA Genomic DNA Isolation Kit (Invitrogen, Carlsbad, CA). The probe used to detect the wild-type codon was 5'-CAGAGACAGCGACCCGG-3', and the probe used to detect the polymorphic codon was 5'-CAGAGACAGTGCACCCG-GAGC-3'. The wild-type probe contained a 5'-FAM reporter dye, whereas the polymorphic probe had a 5'-TET reporter dye. Both probes had a 3'-TAMRA quencher dye. Probes used for allelic discrimination were synthesized by Biosearch Technologies, Inc. (Novato, CA). The forward primer used for PCR was 5'-CGCAGCGGCAAGGC-3', and the reverse primer was 5'-GGTGCT-CAGCTGGTCGTTCT-3' (Life Technologies, Inc., Rockville, MD). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the PCR reaction according to the manufacturer's protocol. DNA (100 ng), primers (900 nM each), and probe (100 nM FAM-tagged or 200 nM TET-tagged) were added to the TaqMan Universal PCR Master Mix in a total volume of 50  $\mu$ L. PCR was carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 62°C for 1 min. Allelic discrimination analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). When necessary, samples that contained the C154T polymorphism were confirmed by *Hha*I restriction digestion of PCR-amplified DNA. C154T abrogates a *Hha*I restriction endonuclease recognition site in the PCR transcript. The wild-type transcript was digested to 50-, 48-, and 21-nucleotide fragments, and the polymorphic allele to 98- and 21-nucleotide fragments.

**Population Prevalence of NKX3.1 (C154T).** For the purpose of determining the frequency of NKX3.1 (C154T) in different racial groups, NKX3.1 genotype was assessed in a cohort of healthy male controls ( $n = 246$ ; age range, 40–79 years) residing in Detroit, Michigan, or in 10 counties in New Jersey who had participated in a population-based case-control study (20).

DNA was extracted from peripheral blood and assessed for NKX3.1 genotype. Blood samples were obtained after informed consent. Cases from this case-control study were not included in the analysis.

**Physicians Health Study Population.** Blood samples were obtained in 1982 from 14,916 men enrolled in the Physicians Health Study. Follow-up questionnaires were completed by 99% of the men through 1995, and follow-up for vital status was 100%. Whenever prostate cancer was diagnosed in the cohort, we sought permission to obtain the medical records to determine stage at diagnosis, tumor grade, and Gleason score. If pathological staging was not available, the case was considered of indeterminate stage unless metastases were clinically evident. We categorized cases as high stage/grade if they were diagnosed at stages C or D, had a Gleason score  $\geq 7$ , or had poor histological differentiation (4). We selected one or two controls at random for each case among the men who returned a blood specimen. Controls were men who had not undergone a radical prostatectomy, had not been diagnosed with prostate cancer at the time of the case diagnosis, and were matched by age and smoking status. DNA was extracted from peripheral blood and sent to E. G. for assay; all assays were performed blinded to case-control status. Samples from 558 cases and 695 controls were assayed. We calculated odds ratios as estimates for the relative risks and 95% confidence intervals from logistic regression models (21), controlling for the matching factors.

## RESULTS

**Occurrence of NKX3.1 R52C Variant in Prostate Cancer Patients and Healthy Men.** To determine the frequency of the NKX3.1 C154T polymorphism in the population, we tested DNA from healthy American white and black men. Prostate cancer incidence is substantially higher in black Americans than in whites, and prostate cancer deaths among blacks also exceed the rate in whites (22–24). We therefore wanted to determine whether there were racial differences in the occurrence of NKX3.1 R52C. We analyzed NKX3.1 genotype in a cohort of 246 healthy men. Overall, 11% of men in the study population were found to carry the NKX3.1 R52C polymorphism. There was a no statistically significant difference in NKX3.1 genotype distribution between the groups of white and black men (Table 1).

To ask whether NKX3.1 genotype influenced prostate cancer risk, we analyzed a nested case-control study of 558 men with prostate cancer from the Physicians Health Study and 695 age-matched controls (4). The results for the total study population (Table 2) showed no statistically significant difference between the two groups. When we analyzed only men who presented with aggressive prostate cancer defined as stage C or D or Gleason score  $\geq 7$ , we found a statistically significant increase in the frequency of NKX3.1 R52C among the cases (relative risk, 1.8; confidence interval, 1.01–3.22). No significant differences between cases and controls were found for nonaggressive cancers or for those men with unknown stage and grade. Because there appeared to be an effect of the polymorphic allele on prostate cancer risk, we sought to investigate whether the variant protein differed in activity from its wild-type counterpart.

**Wild-Type and Variant NKX3.1 Are Differentially Phosphorylated by Protein Kinase C.** The software program Phosphobase v2.0 was used to analyze the NKX3.1 amino acid sequence for possible phosphorylation sites (25). Three consensus sites were identified at serine 48 (44-GRTSSQRQR-52), threonine 117 (115-RLPQTPKQP-123), and threonine 179 (175-RRYKTKRKRQ-184). Serine 48 was a candidate phosphorylation site for calmodulin kinase II, protein kinase

Table 1 NKX3.1 genotype in white and black American men

	NKX3.1 (genotype nt 154), n (%)			
	C/C	C/T	T/T	Total
White	116 (88%)	15	1	132
Black	103 (90%)	9	2	114
Total	219 (89%)	24	3	246

A, and protein kinase C. This site was of interest because it is located in close proximity to the NKX3.1 polymorphism at amino acid 52. Previously, Zannini *et al.* (26) showed that Nkx2.1 could be phosphorylated by protein kinase C. Wild-type NKX3.1 fused with maltose-binding protein was phosphorylated *in vitro* by protein kinase C (Fig. 1A). In addition to NKX3.1 phosphorylation, a minor level of protein kinase C autophosphorylation was present, represented by the 80-kDa band. Phosphobase v2.0 also identified four consensus protein kinase C phosphorylation sites in the amino acid sequence of the maltose-binding protein affinity tag. However, protein kinase C did not phosphorylate maltose-binding protein alone, suggesting that phosphorylation of the fusion protein was specific for the NKX3.1

Table 2 Relative risk of prostate cancer according to the CGC→TGC polymorphism in NKX3.1

NKX3.1 genotype	No. cases (%)	No. controls (%)	RR <sup>a</sup>	95% CI
Overall cancer				
CC	499 (89.4)	637 (91.7)	1.00	Reference
CT	57 (10.2)	55 (7.9)	1.32	0.90–1.95
TT	2 (0.4)	3 (0.4)	0.85	0.14–5.11
CT + TT			1.30	0.89–1.90
Nonaggressive cancer <sup>b</sup>				
CC	234 (90.4)	285 (89.6)	1.00	Reference
CT	25 (9.7)	31 (9.8)	0.98	0.56–1.71
TT	0	2 (0.6)	0	
CT + TT			0.92	0.53–1.60
Aggressive cancer <sup>b</sup>				
CC	229 (88.8)	313 (93.4)	1.00	Reference
CT	27 (10.5)	21 (6.3)	1.76	0.97–3.19
TT	2 (0.8)	1 (0.3)	2.73	0.25–30.33
CT + TT			1.80	1.01–3.22
Unknown aggressiveness				
CC	36 (87.8)	39 (92.9)	1.00	Reference
CT	5 (12.2)	3 (7.1)	1.81	0.40–8.10
TT	0	0	0	
CT + TT			1.81	0.40–8.10

<sup>a</sup> RR, relative risk; CI, confidence interval.

<sup>b</sup> Aggressive cancers were those cases identified at presentation as stages C or D or Gleason score  $\geq 7$ .

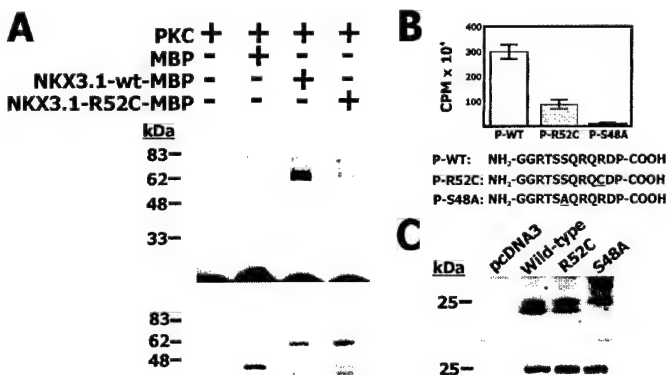


Fig. 1. Protein kinase C preferentially phosphorylates wild-type NKX3.1. A, purified maltose-binding protein (MBP) or NKX3.1 fusion proteins (200 ng) were used as substrates in kinase reactions with protein kinase C (10 ng) and 0.11  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After the kinase reactions, samples were electrophoresed on a denaturing 10–20% gradient polyacrylamide gel. Phosphorylated proteins were identified by autoradiography (top). Western blotting with rabbit antiserum to maltose-binding protein (5  $\mu$ g) was used to control for protein loading (bottom). B, peptides (30  $\mu$ g) representing amino acids 43–54 of wild-type (P-WT), R52C (P-R52C), or S48A (P-S48A) NKX3.1 were used in an *in vitro* kinase assay with 10 ng of protein kinase C and 0.11  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After the kinase reaction, samples were transferred to phosphocellulose discs and washed, and the incorporated radioactivity was measured as cpm by liquid scintillation counting. Amino acid sequences of the peptides are shown below the graph. C, LNCaP cells were transfected with vectors expressing wild-type, R52C, or S48A NKX3.1 with an NH<sub>2</sub>-terminal FLAG tag. The cells were treated with R1881, and 48 h later, the cells were exposed to 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were lysed, and NKX3.1 was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates were electrophoresed, and radiolabeled proteins were visualized by autoradiography (top). Western blotting with an anti-FLAG antibody (20  $\mu$ g) was used to control for protein loading (bottom).

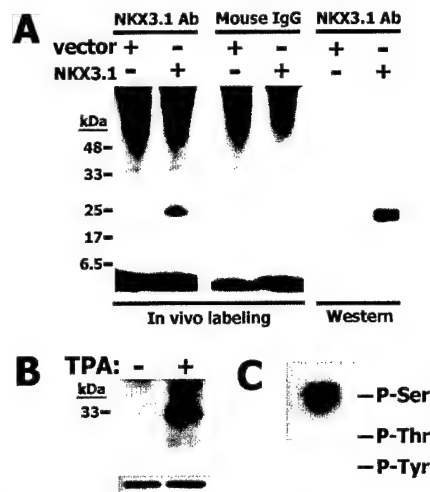


Fig. 2. NKX3.1 is phosphorylated *in vivo*. A, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression vector or empty vector. Forty-eight h post-transfection, cells were treated with 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were then lysed, and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antiserum or mouse IgG. Immunoprecipitates were electrophoresed by SDS-PAGE, and phosphorylated protein was visualized by autoradiography. The same antibody used for immunoprecipitation of NKX3.1 was used in a Western blot of lysates from cells transfected with either empty vector or NKX3.1 expression vector. B, LNCaP cells were treated with R1881, and 48 h later the cells were exposed to 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were then treated with or without TPA (100 nM) for an additional 30 min. Cells were lysed, NKX3.1 was immunoprecipitated with an anti-NKX3.1 antibody and electrophoresed, and radiolabeled proteins were visualized by autoradiography (top). Western blotting with an anti-NKX3.1 antibody (1.5  $\mu$ g) was used to control for protein loading (bottom). C, endogenous radiolabeled NKX3.1 was excised from a polyacrylamide gel, eluted, and treated with 0.15 mg/ml trypsin. The digested protein was hydrolyzed with 6 N HCl for 1 h at 105°C. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis. The identity of the phosphorylated amino acids was determined by autoradiography and comparison with phosphoamino acid standards.

moiety. In contrast, protein kinase C phosphorylation of NKX3.1 R52C was noticeably decreased relative to phosphorylation of wild-type NKX3.1 (Fig. 1A). The results of the Western blotting with anti-maltose-binding protein shown in the bottom panel of Fig. 1A indicate that equal amounts of fusion protein with either wild-type or NKX3.1 R52C were present in the reaction.

To confirm that the arginine-to-cysteine variation specifically affected phosphorylation by protein kinase C, synthetic peptide substrates representing amino acids 43–54 of NKX3.1 were used as protein kinase C substrates (Fig. 1B). Relative phosphorylation of peptide from wild-type NKX3.1 was 3-fold higher than for the NKX3.1 R52C peptide. Phosphorylation of peptide with an alanine replacing serine 48 was decreased 33-fold relative to the wild-type sequence. To determine the effects of amino acid alterations on the serine 48 phosphorylation *in vivo*, NKX3.1, NKX3.1 R52C, or NKX3.1 S48A expression vectors with a FLAG tag were used to transfect LNCaP prostate cancer cells that expressed endogenous NKX3.1 as well (7). The level of phosphorylation in the cells transfected with the NKX3.1 R52C variant was half that in the cells transfected with the wild-type protein. The mutation of serine 48 to alanine essentially eliminated *in vivo* phosphorylation of Flag-tagged NKX3.1 (Fig. 1C), providing evidence that serine 48 is a major *in vivo* phosphoacceptor.

TSU-Pr1 cells, which do not express NKX3.1, were used for [<sup>32</sup>P]P<sub>i</sub> labeling of COOH-terminal truncated NKX3.1 (Fig. 2A). Western blotting confirmed that only exogenous NKX3.1 protein was detected in the cells (Fig. 2A). Endogenous full-length NKX3.1 in LNCaP cells was also phosphorylated *in vivo*, and the level of phosphorylation was increased by the presence of 100 nM TPA (Fig. 2B), suggesting that NKX3.1 was phosphorylated *in vivo* by a TPA-induced kinase, such as protein kinase C. Protein kinase C did not affect levels of endog-

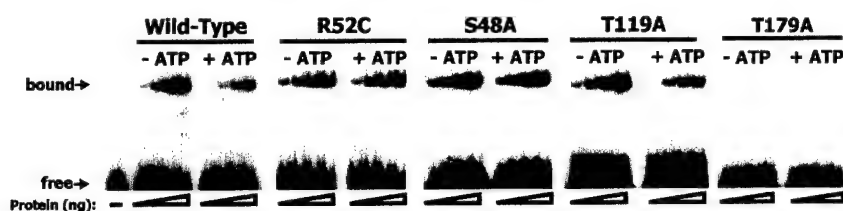


Fig. 3. R52C polymorphism affects phosphorylation-regulated DNA binding. Purified fusion proteins (200 ng) were treated with protein kinase C (10 ng) in the presence or absence of cold ATP. After protein kinase C treatment, the proteins (2, 5, 10, or 25 ng) were used in gel shift assays with a radiolabeled NKX3.1 consensus DNA binding sequence. Protein-bound DNA was separated from free probe by 8% native PAGE, and the results were visualized by autoradiography.

enous NKX3.1 as determined by Western blotting (Fig. 2B). Phosphoamino acid analysis of the radiolabeled endogenous protein in LNCaP cells indicated that NKX3.1 was phosphorylated only at serine (Fig. 2C). Similar phosphoamino acid analysis results were obtained when we labeled exogenous NKX3.1 in transfected TSU-Pr1 cells (data not shown). Moreover, no phosphorylation was seen in several attempts to label NKX3.1 S48A *in vivo*.

**Phosphorylation at Serine 48 Regulates *in Vitro* NKX3.1 DNA Binding.** Recombinant purified NKX3.1 fusion proteins were treated with protein kinase C in the presence or absence of ATP, and the proteins were included in gel shift assays with a radiolabeled NKX3.1 high-affinity DNA binding sequence (15). Phosphorylation of wild-type NKX3.1 decreased the apparent binding affinity of the protein for the consensus sequence by 3-fold relative to the nonphosphorylated protein (Fig. 3). However, the DNA binding of NKX3.1 R52C was not noticeably altered after treatment with protein kinase C in the presence of ATP. Similarly, NKX3.1 S48A lost regulation of DNA binding by phosphorylation (Fig. 3). To compare the effects of phosphorylation at two other consensus protein kinase C phosphorylation sites on DNA binding, either threonine 119 or threonine 179 was mutated to alanine. Protein kinase C treatment of NKX3.1 T119A yielded DNA binding data similar to those for wild-type NKX3.1 (Fig. 3). Interestingly, the T179A mutation, located in the homeodomain, abrogated NKX3.1 DNA binding (Fig. 3).

**Effect of Polymorphism on NKX3.1 Transcriptional Activity.** NKX3.1 and the heart-specific NK family protein NKX2.5 have very similar *in vitro* properties. The activities of these proteins are mediated largely by the homeodomains, which are nearly identical in their three major homeodomain helices and coincide at 39 of 60 amino acids. Both proteins bind SRF (16, 27), and NKX2.5 was shown to bind SRF *via* the homeodomain (27). Because of similarities in their homeodomain primary structure, NKX3.1 is likely also to bind SRF *via* the homeodomain. To assess NKX3.1 coactivation of SRF, we used a transcription assay with a reporter construct under control of the SMGA promoter, similar to the transactivation experiment reported by Carson *et al.* (16). We found that human NKX3.1 can act as a coactivator for SRF activation of transcription from the SMGA promoter, similar to previously published results for murine Nkx3.1 (16). In general, the presence of NKX3.1 resulted in a 3–5-fold increase in SMGA promoter activity. We compared full-length wild-type NKX3.1 expression vector with mutant constructs that coded for NKX3.1 R52C and NKX3.1 S48A protein variants. As a control we used an expression construct with NKX3.1 in reverse orientation that coded for no protein. The three coding constructs had similar levels of NKX3.1 protein expression and similar levels of SRF coactivation. Treatment of the cultures with 100 nM TPA, which had been shown to cause phosphorylation of NKX3.1 in culture, had a minimal inhibitory effect on the activity of each construct, but did not differentially affect the coactivation by mutant and wild-type constructs (Table 3).

## DISCUSSION

A common polymorphism in the prostate-specific homeoprotein may have an effect on prostate cancer pathogenesis as risk factor for aggressive disease. A tumor suppressor function of NKX3.1 has been suggested by studies of gene-targeted mice. Targeted disruption of murine *Nkx3.1* suggested that the gene exerts growth suppression and differentiating effects on prostatic epithelium (9, 28). Importantly, animals heterozygous for loss of *Nkx3.1* demonstrated histological disarray of the prostate and bulbourethral gland, suggesting that haploinsufficiency was dominant. Because the murine gene lacks an amino acid similarity at arginine 52 and lacks the protein kinase C phosphorylation site at serine 48, it is hard to predict the role of this putative regulatory region in the mouse.

We found that the R52C polymorphism occurs with similar frequency among whites and blacks in the United States. Prostate cancer is more common among blacks than whites in the United States, with a higher mortality among blacks than whites (22–24). Therefore, it does not appear that disparities in the frequency of NKX3.1 C154T contribute to the difference in prostate cancer between the races. Approximately 5–10% of prostate cancer is inherited in a Mendelian fashion that has been traced to at least three susceptibility loci on chromosomes 1, X, and 17 (29–32).

The occurrence of sporadic prostate cancer, however, is likely to be influenced subtly by many genes that affect susceptibility. Much attention has been directed to variations in the polyglutamine tract in the NH<sub>2</sub> terminus of the androgen receptor. Shorter polyglutamine repeat lengths are associated with increased androgen receptor activity and more aggressive prostate cancer (4, 5, 33). Other genetic factors that may have a subtle effect on prostate cancer risk in the general population include the vitamin D receptor (34–36), *CYP17* (37), 5 $\alpha$ -reductase A49T (38), and glutathione S-transferase  $\theta$  (39). The NKX3.1 C154T genotype may be one of those subtle genetic influences on prostate cancer risk, in particular for aggressive disease.

The NKX3.1 C154T polymorphism appears to affect a region of the protein that can affect DNA binding. The exact role of the region containing amino acids 48–52 has not been determined, but it is clear that the region is important for phosphorylation. Homeoproteins are known to undergo posttranslational modification by phosphorylation. Homeoprotein phosphorylation has been shown to affect protein-protein interactions (40), subcellular localization (41), DNA binding affinity (42), and transcriptional activity (43). Generally, these effects

Table 3 SRF coactivation assay of NKX3.1 and mutant constructs

Construct	Fold SRF coactivation	
	–TPA	+TPA
Control	1.06	NT <sup>a</sup>
NKX3.1	4.4	3.7
NKX3.1 R52C	5.8	4.6
NKX3.1 S48A	6.8	5.6

<sup>a</sup> NT, not tested.



have been attributed to electrostatic repulsion or a conformational change in the protein (44). Members of the NK family of homeoproteins have been shown to undergo phosphorylation. The kinases responsible for phosphorylating NK-class homeoproteins include casein kinase II (43), MST2 kinase (45), extracellular signal-regulated kinase (46), homeodomain-interacting protein kinase (47), protein kinase A (48), and protein kinase C (26).

Although we believe that the cellular activity of NKX3.1 R52C is different from that of the wild type, the precise impact of the polymorphism on NKX3.1 function is unclear. Although relatively little is known about the protein interactions of NKX3.1, the NK family member NKX2.5 has been characterized more extensively and has been shown to interact with DNA and with at least two other transcription factors, GATA-4 and SRF (27, 49, 50). Moreover, the protein-protein interactions of NKX3.1 are mediated by the homeodomain as well (27, 49, 50). It is entirely possible that NKX3.1, like NKX2.5, undergoes multiple interactions that are involved in manifestation of its biological effects. However, the analogy between the two homeoproteins has yet to be proved. It should be remembered that although the two proteins share nearly identical homeodomain sequences, they have very little amino acid identity in the NH<sub>2</sub>- and COOH-terminal regions.

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## Expression of NKX3.1 in Normal and Malignant Tissues

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**BACKGROUND.** NKX3.1, a member of the NK-class of homeodomain proteins, is expressed primarily in the adult prostate and has growth suppression and differentiating effects in prostate epithelial cells.

**METHODS.** We performed immunohistochemical analysis for NKX3.1 and PSA expression in 4,061 samples included in a tissue microarray of a broad spectrum of human cancers and normal tissues.

**RESULTS.** NKX3.1 expression was seen in prostate epithelial cells, prostate cancer, normal testis, 9% of primary and 5% of metastatic infiltrating ductal breast carcinoma, and 27% of primary and 26% of metastatic infiltrating lobular breast carcinoma. In a cohort of 474 primary breast cancers with median follow-up over 62.5 month survival, we found no effect of NKX3.1 expression on prognosis. NKX3.1 expression was more restricted than the spectrum of prostate specific antigen expression.

**CONCLUSIONS.** Expression of NKX3.1 is highly restricted and is found primarily in benign and malignant prostatic epithelial cells and also in normal testis and lobular carcinoma of the breast. *Prostate* 9999: 1–7, 2003. © 2003 Wiley-Liss, Inc.

**KEY WORDS:** prostate cancer; breast cancer; lobular carcinoma; prostate specific antigen

### INTRODUCTION

NKX3.1 is an androgen-regulated NK-class homeobox gene with expression in adult mice and humans localized primarily in the prostate [1–4]. The NKX3.1 gene has been conserved during evolution; the murine and human proteins share 63% amino acid identity. The human NKX3.1 has been mapped to chromosome 8p21 [2,5], and has been located in a region of the chromosome deleted in approximately 85% of prostate cancer [6–10]. However, no tumor-specific mutations of the NKX3.1 protein-coding region have been identified by genetic analysis of human prostate cancer samples [5]. Nevertheless loss of NKX3.1 expression was found to correlate with prostate cancer progression [11]. Decreased expression of NKX3.1 may have a role in prostate cancer pathogenesis since heterozygous *Nkx3.1* gene targeted mice displayed a phenotype of prostatic hyperplasia suggesting that in humans NKX3.1 haploinsufficiency may be dominant [4].

NKX3.1 also has tumor suppressor properties and is proapoptotic in vitro (Giuffrida and Gelmann, submitted for publication) and, therefore, may be an appropriate target for the development of therapeutic strategies aimed at enhancing its activity.

The expression of NKX3.1 in normal tissues is highly restricted. In mice, expression of *Nkx3.1* mRNA is seen only in prostatic lobes and the bulbourethral gland [4].

Abbreviations used: PSA, prostate-specific antigen

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In humans, *NKX3.1* mRNA is seen in prostate and testis [2] and expression of NKX3.1 protein can be found only in prostate epithelial cells, testis, bronchial mucous glands, and rare ureteral cells [11]. Because of its highly restricted expression pattern NKX3.1 could serve as a marker to identify prostatic carcinoma. Also, because of its tumor suppressor properties, NKX3.1 expression could have prognostic implications. To define the spectrum of NKX3.1 expression in tumor tissues we analyzed a tissue microarray containing a wide spectrum of human tumor tissues. We compared the expression of NKX3.1 to the expression of PSA, a widely used marker for prostatic adenocarcinoma. We also found that NKX3.1 was expressed in some breast cancer tissues, but not in normal breast tissue. We also analyzed the prognostic significance of NKX3.1 expression in a cohort of primary breast cancer specimens included in a breast cancer tissue microarray.

## MATERIALS AND METHODS

### Immunohistochemistry

Standard indirect immunoperoxidase procedures were carried out in combination with an antiserum to recombinant human NKX3.1 as previously described [11] and a monoclonal antibody to PSA (ER-PR8 at a dilution of 1:400, DAKO, Glostrup, Denmark). Diaminobenzidine was used as a chromagen for PSA detection. Staining and scoring for NKX3.1 staining intensity has been described [11]. Samples were scored as NKX3.1 positive by EPG based on intensity and uniformity of nuclear staining in malignant cells as described previously. Samples were scored as PSA positive by LB based on cytoplasmic staining.

### Tumor Microarrays

These have been described previously [12,13]. The Tissue Microarray contained 4,061 analyzable samples of malignant and normal tissues representative of a wide range of samples (Table I). All specimens on the tissue microarrays utilized in this study had been processed at the Institute for Pathology, University of Basel, with standard overnight fixation in 4% buffered formalin and subsequent paraffin embedding. One core was taken from each donor block. Cancers where there was only a single faintly staining sample were not considered to have significant levels of expression. The Breast Prognostic Microarray was assembled from primary breast cancer specimens as previously described [14].

## RESULTS

We analyzed 4,061 human tissues and tumors in a tissue microarray. Significant NKX3.1 staining, defined

**TABLE I. List of Tissues in 4,061-Sample Tissue Microarray**

Tissue	N =
Adenomatoid tumor	9
Adrenal gland, adenoma	14
Adrenal gland, carcinoma	6
Adrenal gland, normal tissue	10
Adrenal, pheochromocytoma	30
Anus, normal tissue	4
Anus, squamous cell carcinoma	6
Appendix, adenocarcinoma	1
Appendix, carcinoid	11
Appendix, normal tissue	7
Benign salivary gland tumors of the skin	20
Blood vessel, normal tissue	10
Blood vessel, capillary hemangioma	11
Blood vessel, hemangiopericytoma	8
Brain, astrocytoma	33
Brain, cerebral cortex, normal tissue	22
Brain, craniopharyngeoma	3
Brain, ependymoma	11
Brain, glioblastoma multiforme	45
Brain, medulloblastoma	5
Brain, meningioma	43
Brain, oligodendroglioma	20
Breast cancer, apocrine carcinoma	4
Breast cancer, cribriform carcinoma	8
Breast cancer, cribriform, metastases	1
Breast cancer, ductal carcinoma in situ	32
Breast cancer, invasive ductal carcinoma	55
Breast cancer, invasive ductal, metastasis	390
Breast cancer, invasive lobular carcinoma	31
Breast cancer, invasive lobular, metastasis	56
Breast cancer, medullary carcinoma	45
Breast cancer, mucinous carcinoma	12
Breast cancer, mucinous, metastases	1
Breast cancer, papillary carcinoma	7
Breast cancer, papillary, metastases	1
Breast cancer, tubular carcinoma	6
Breast, normal tissue	8
Breast, phylloides tumor	17
Colon adenoma, mild dysplasia	24
Colon adenoma, moderate dysplasia	20
Colon adenoma, severe dysplasia	21
Colon, adenocarcinoma	331
Colon, adenocarcinoma metastasis	25
Colon, normal tissue	10
Dermatofibrosarcoma protuberans	4
Endometrioid stroma sarcoma	2
Endometrium, carcinoma	123
Endometrium, carcinoma metastasis	3
Endometrium, normal tissue	8
Epithelioid hemangioma	2
Esophagus, adenocarcinoma	4
Esophagus, normal tissue	7
Esophagus, squamous cell carcinoma	15
Esthesioneuroblastoma	2
Fat, lipoma	31

(Continued)

TABLE I. (Continued)

Tissue	N=
Fat, liposarcoma	20
Fat, normal tissue	9
Fetal adrenal glands, normal tissue	6
Fetal heart, normal tissue	8
Fetal kidney, normal tissue	5
Fetal liver, normal tissue	8
Fetal lung, normal tissue	9
Gall bladder, adenocarcinoma	12
Gall bladder, adenocarcinoma metastasis	2
Gall bladder, normal tissue	8
Gastrointestinal stroma tumor	13
Glomus tumor	9
Granulosa cell tumor	13
Histiocytoma, benign	11
Kaposi sarcoma	16
Kidney, normal tissue	12
Kidney, renal cell carcinoma, chromophobe	10
Kidney, renal cell carcinoma, clear cell	92
Kidney, renal cell carcinoma, metastasis	1
Kidney, renal cell carcinoma, papillary	43
Larynx, squamous cell carcinoma	22
Larynx, squamous cell carcinoma, metastasis	3
Leukemia, acute myeloid	2
Leukemia, chronic myeloid	4
Liver, hepatocellular carcinoma	46
Liver, normal tissue	8
Lung cancer adenocarcinoma metastasis	8
Lung cancer, adenocarcinoma	30
Lung cancer, large cell anaplastic carcinoma	35
Lung cancer, small cell carcinoma	18
Lung cancer, squamous cell carcinoma	32
Lung cancer, squamous cell carcinoma, metastasis	7
Lung, carcinoid	11
Lung, normal tissue	8
Lymph node, normal tissue	9
Lymphoma, Hodgkin, mixed cellularity	31
Lymphoma, Hodgkin, nodular sclerosis	16
Lymphoma, non Hodgkin	101
Mesenchymoma, malignant	11
Mesothelioma, malignant	9
Mixed Mullerian tumor, malignant	5
Nerve, ganglioneuroma	7
Nerve, neurofibroma	31
Nerve, normal tissue	5
Nerve, paraganglioma	15
Nerve, primitive neuroectodermal tumor (PNET)	17
Nerve, Schwannoma	40
Nerve, Schwannoma malignant	8
Nevus cell nevus	24
Oral cavity, normal tissue	3
Oral cavity, squamous cell carcinoma	18
Oral cavity, squamous cell carcinoma, metastasis	33
Ovarian cancer, endometroid	38
Ovary cancer, metastasis	3
Ovary cancer, mucinous	16

TABLE I. (Continued)

Tissue	N=
Ovary cancer, nos	26
Ovary cancer, rare types	5
Ovary cancer, serous	48
Ovary, Brenner tumor	7
Ovary, normal tissue	8
Pancreas, adenocarcinoma	25
Pancreas, adenocarcinoma metastasis	2
Pancreas, ampulla of Vater, adenocarcinoma	3
Pancreas, ampulla of Vater, metastasis	2
Pancreas, normal tissue	7
Parathyroid, adenoma	21
Parathyroid, carcinoma	1
Parathyroid, normal tissue	8
Penile squamous cell carcinoma, metastasis	2
Placenta	9
Prostate cancer, hormone refractory	11
Prostate cancer, metastasis	9
Prostate cancer, untreated	65
Prostate, normal tissue	6
Salivary gland, acinus cell carcinoma	7
Salivary gland, adenoid cystic carcinoma	21
Salivary gland, adenolymphoma	33
Salivary gland, adenoma	8
Salivary gland, lymphoepithelial carcinoma	1
Salivary gland, metastasis	3
Salivary gland, mucoepidermoid carcinoma	2
Salivary gland, normal tissue	8
Salivary gland, other carcinoma types	13
Salivary gland, pleomorphic adenoma	33
Sarcoma, alveolar	1
Sarcoma, angiosarcoma	2
Sarcoma, epithelioid	2
Sarcoma, fibrosarcoma	7
Sarcoma, malignant fibrous histiocytoma	19
Sarcoma, synovial cell	3
Skin, basal cell carcinoma	29
Skin, melanoma	70
Skin, Merkel cell carcinoma	2
Skin, normal tissue	7
Skin, squamous cell carcinoma	27
Skin, squamous cell carcinoma, metastasis	3
Small intestine, adenocarcinoma	9
Small intestine, carcinoid	7
Small intestine, metastasis	1
Small intestine, normal tissue	10
Smooth muscle, leiomyoblastoma	8
Smooth muscle, leiomyosarcoma	46
Smooth muscle, myoma	15
Smooth muscle, normal tissue	9
Stomach cancer, metastasis	5
Stomach, adenocarcinoma	118
Stomach, carcinoid	1
Stomach, normal tissue	11
Striated muscle, normal tissue	9
Striated muscle, rhabdomyosarcoma	10

(Continued)

TABLE I. (Continued)

Tissue	N =
Tendon sheath, giant cell tumor	35
Testis, Leydig cell tumor	3
Testis, non-seminomatous germ cell tumors	74
Testis, normal tissue	7
Testis, seminoma	37
Thymoma	23
Thymus, normal tissue	9
Thyroid cancer, anaplastic	8
Thyroid cancer, follicular	32
Thyroid cancer, medullary	14
Thyroid cancer, metastasis	3
Thyroid cancer, papillary	28
Thyroid, adenoma	29
Thyroid, normal tissue	9
Unknown primary, adenocarcinoma	17
Urinary bladder cancer, squamous cell carcinoma	11
Urinary bladder, adenocarcinoma	6
Urinary bladder, inverted papilloma	2
Urinary bladder, noninvasive cancer (pTa)	105
Urinary bladder, normal tissue	5
Urinary bladder, sarcomatoid cancer	8
Urinary bladder, small cell cancer	8
Urinary bladder, transitional cell carcinoma (pT2-4)	40
Uterine cervix, adenocarcinoma	3
Uterine cervix, carcinoma in situ	10
Uterine cervix, normal tissue	6
Uterine cervix, squamous cell carcinoma	34
Uterine cervix, squamous cell carcinoma, metastasis	3
Vagina, normal tissue	4
Vagina, squamous cell carcinoma	7
Vulva, squamous cell carcinoma	40
Total	4,061

as finding detectable nuclear "grade 1" staining in a tissue type, was seen in the samples listed in Table II. Table II contains only tissues of which more than a single sample had positive staining and tissues with overall staining frequency for either NKX3.1 or PSA of greater than 10% except for infiltrating ductal carcinoma of the breast. Staining was seen in all categories of prostatic tissues. As we have observed previously, decreased frequency of staining prostate cancer correlated with cancer progression [11]. We also detected NKX3.1 staining in normal testis, metastases of infiltrating lobular breast carcinoma (28%), and metastases of infiltrating ductal breast carcinoma (5%) (Table II). There was no NKX3.1 expression in normal breast, endometrium, or salivary glands. Nuclear NKX3.1 staining of single samples of other tissue types was seen in only 11 instances.

In this sample set, NKX3.1 expression was more restricted in tissue-specificity than PSA using the positivity criteria for both markers as described in Materials and Methods (Table II). The spectrum of PSA staining extended to parathyroid, thyroid, salivary gland, and astrocytoma.

Because of the novel finding of NKX3.1 expression in breast cancer, particularly lobular carcinoma of the breast, we interrogated a tissue microarray of primary breast cancer samples to confirm the finding of NKX3.1 expression in breast cancer and to determine if NKX3.1 expression influenced breast cancer prognosis. Examples of NKX3.1 expression in breast cancer specimens are shown in Figure 1. Among 494 samples analyzed in the tissue microarray, approximately 26% of primary lobular carcinoma specimens, a similar proportion to the metastases, expressed NKX3.1 (Table III). Other histologic types of breast cancer display less than 10% of samples with staining for NKX3.1. The significance of NKX3.1 expression in breast cancer is unknown. Analysis comparing those with and without NKX3.1 expression showed no influence on cause-specific or overall survival.

## DISCUSSION

We have confirmed that expression of the prostate homeoprotein NKX3.1 is highly restricted in normal human tissues and is seen in prostate epithelium and testis. The limited number of samples from hormone refractory (11) and metastatic prostate cancer (9) had a lower fraction of NKX3.1 staining, consistent with our published observations that NKX3.1 expression decreases with prostate cancer progression [11]. We also confirmed the staining of normal seminiferous tubules with antibody to NKX3.1. No expression of NKX3.1 was seen in samples from testicular carcinoma. We have also reported that bronchial mucous glands and rare ureteral cells express NKX3.1. The nature of the tissues in the microarray did not provide sufficient material to confirm these observations. However, we have recently analyzed NKX3.1 expression in bronchial mucous glands of both men and women and found expression of the protein in those glands independently of gender (E.P. Gelmann and B. Singh, unpublished observations).

In contrast to the limitations of tissue microarrays to contain representative fragments of normal anatomic structures, tissue microarrays can be very useful in the analysis of large numbers of relatively homogeneous tumors. To exploit this we have analyzed a tissue microarray with representative tissues from a wide spectrum of human cancers. Interestingly, we observed no expression of NKX3.1 in malignant nonprostate cells that may correspond to normal tissues where NKX3.1 is



TABLE II. NKX3.1 and PSA Staining in 4,061 Cancer Microarray

Tissue	N=	NKX3.1 staining			NKX3.1 % +ive	PSA staining			PSA % +ive
		0	1	2		1	2	3	
Prostate cancer, hormone refractory	11	8	2	1	27.27	1	4	0	45.45
Prostate cancer, metastases	9	5	0	4	44.44	0	1	8	100.00
Prostate cancer, untreated	65	20	16	27	66.15	1	3	60	98.46
Prostate, normal tissue	6	0	3	1	66.67	1	0	5	100.00
Breast cancer, ductal carcinoma metastases	380	361	16	3	5.00	0	2	0	0.53
Breast cancer, lobular carcinoma metastases	56	40	14	2	28.57	0	0	0	0.00
Astrocytoma	33	33	0	0	0.00	4	0	0	12.12
Parathyroid, adenoma	21	21	0	0	0.00	5	0	0	23.81
Parathyroid, normal tissue	8	8	0	0	0.00	0	3	0	37.50
Salivary gland, adenolymphoma	33	33	0	0	0.00	3	21	0	72.73
Salivary gland, adenoma	8	8	0	0	0.00	1	0	0	12.50
Salivary gland, normal tissue	8	8	0	0	0.00	3	0	0	37.50
Testis, normal tissue	7	3	4	0	57.14	0	0	0	0.00
Thyroid, follicular cancer	32	32	0	0	0.00	8	2	0	31.25

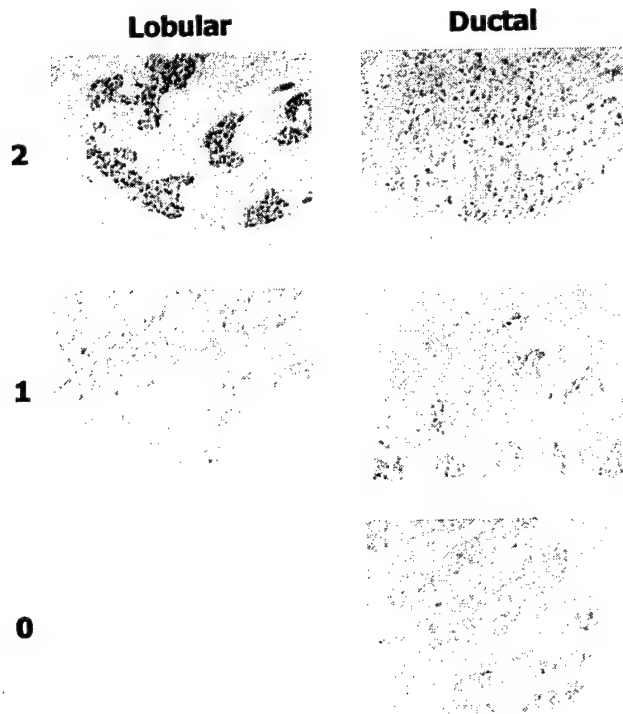


Fig. 1. Representative NKX3.1 immunohistochemical staining of primary breast cancer samples in the Breast Cancer Prognostic Array are shown. Digital microscopy images were made with a 10 × objective and processed identically. Note that tissues that were scored positively (2 or 1) had uniform nuclear staining. A ductal carcinoma sample is shown as an example of negative staining that has some cytoplasmic and perinuclear nonspecific staining.

expressed. In addition to absence of NKX3.1 staining in testicular cancer, we saw no staining in transitional cell carcinoma or pulmonary adenocarcinoma. A subset of breast cancer tissues stained positively with NKX3.1 antibody. The frequency of staining and preference for lobular carcinoma was seen in separate sample sets of metastatic and primary cancer. On analysis of 494 patients with primary breast cancer and a median 62.5 months survival, we found no effect of NKX3.1 expression on overall survival.

NKX3.1 expression was found more commonly in lobular than ductal carcinoma. Current approaches to combined modality therapy of breast cancer can abrogate differences in prognosis reflected by molecular markers. However, expression of NKX3.1 joins the list of molecular markers with differential expression between lobular and ductal carcinoma [15–17]. Whether these discriminating markers reflect biological differences that can be exploited in the development of less toxic therapies remains to be seen.

Although NKX3.1 expression was shown to be stimulated by androgens in LNCaP prostate cancer cells, the expression of NKX3.1 in female tissues—both breast cancer and pulmonary mucous glands (our unpublished observations), suggests that NKX3.1 expression does not require optimal androgen action [2]. This fact was recently underscored by the report of a prostate cancer cell line that expressed NKX3.1 mRNA but no androgen receptor mRNA as determined by polymerase chain reaction of reverse transcriptase-generated cDNA [18].

PSA staining has been previously reported in tissues other than prostate, such as breast and salivary gland, although immunoreactivity assays with homogenized



TABLE III. NKX3.1 Staining of Breast Cancer Tissue Microarray

Tumor type	N	NKX3.1 staining score			% NKX3.1 positive
		0	1	2	
Apocrine	4	4	0	0	0
Clear cell	4	4	0	0	0
Cribiform	10	9	0	1	10
Ductal	368	335	28	5	9
Lobular	63	47	13	3	26
Medullary	16	15	1	0	6
Metaplastic	1	1	0	0	
Mucinous	12	11	1	0	8
Neuroendocrine	1	1	0	0	
Papillary	7	7	0	0	0
Small cell	1	1	0	0	
Tubular	7	7	0	0	0
Total	494	442	43	9	10.5

tissue often failed to confirm the presence of bona fide PSA in these tissues [19]. PSA mRNA was detected in prostate, salivary gland, pancreas, and uterus [20]. Expression of PSA mRNA in thyroid oxyphilic cells has also been reported [21]. The finding of PSA in cells of parathyroid origin and in astrocytoma is novel.

### CONCLUSIONS

NKX3.1 expression was more restricted than PSA expression and found in a limited spectrum of tissues, most significantly the prostate. Although NKX3.1 expression was seen in normal ureter and pulmonary mucous glands [11], no expression was seen in transitional cell carcinoma and pulmonary adenocarcinoma. On the other hand, no expression was seen in normal breast, but a substantial fraction of lobular carcinomas had low levels of NKX3.1 expression. Expression of NKX3.1 in breast cancer had no obvious impact on prognosis.

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### **Searching for the Gatekeeper Oncogene of Prostate Cancer**

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## **Abstract**

Prostate cancer is a common malignancy that has a heterogeneous etiology and a variable outcome. Nearly all prostatic adenocarcinoma results from androgen-dependent tumor promotion. However, the cause of prostate cancer initiation is not well understood and only a few of the target oncogenes activated during prostate cancer initiation have been identified. Prostate cancer risk is strongly influenced by family history. Several genetic loci have been found to cosegregate with prostate cancer occurrence in high-risk families. Some candidate oncogenes that map to these loci have been implicated by the identification of mutations in high-risk kindreds. However, the roles of the putative oncogene products in the biochemical pathways that mediate carcinogenesis remain obscure and their influence on cancer etiology has yet to be supported by gene targeting experiments in mice. Moreover, the genes that have been implicated in hereditary prostate cancers do not appear to be mutated in sporadic cancers. Karyotypic and loss of heterozygosity analysis of sporadic prostate cancers have identified 8p, 10q, and 17p as the loci most often disrupted. Candidate oncogenes have been identified at each of these regions. Additional genes with pathogenic significance in prostate cancer have been identified by analysis of cDNA microarrays comparing benign and malignant prostate tissue, by differential genetic analysis of benign and malignant prostatic epithelium, and by induction of experimental prostate cancer in genetically engineered mice.

Prostate cancer, like other solid tumors, results from the accumulation of mutations and other genetic and epigenetic events that occur in a semi-ordered fashion to confer the malignant phenotype on normal cells. By analogy with other cancers it is likely that a gatekeeper mutation is required for the evolution of malignant change in prostatic epithelial cells [1]. The significance of the gatekeeper gene in initiation and maintenance of the malignant phenotype has been demonstrated by the profound therapeutic effects of imatinib mesylate (Gleevec®), an ABL kinase inhibitor, in both chronic and acute phase leukemias that contain translocations of the gatekeeper BCR/ABL oncogene, in gastrointestinal stromal tumors with activate *KIT*, and in myeloproliferative syndromes with activated platelet-derived growth factor receptor-B [2-5]. A hallmark of gatekeeper genes is the high frequency of their disruption in specific malignancies. In particular, expression of normal APC, coded by a gene on chromosome 5q, is lost in a high percentage of colon cancers that evolve from polyps and adenomas [6], and in the absence of *APC* mutations other genes in the pathway whose products interact with APC, such as the  $\beta$ -catenin gene, are mutated [7,8].

The identification of chromosomal loci that cosegregate with familial predisposition to cancers has facilitated the identification of some gatekeeper genes. For example, *APC*, is the gene targeted on chromosome 5q in familial adenomatous polyposis, an inherited condition that predisposes to colonic neoplasia [9-11]. The *VHL* gene that is disrupted in nearly all renal cell carcinomas is also the target gene in the von Hippel-Lindau Syndrome [12]. Since risk for prostate cancer is influenced profoundly by family history it seems reasonable to expect that some families have inherited mutations in prostate gatekeeper genes. Studies of a large number of kindreds have identified four candidate loci that may be contributory to prostate cancer risk. Because of the large number of sporadic cases of prostate cancer among older men, it is difficult

to determine whether prostate cancer was caused by the presence of an inherited genetic trait or not. For this reason the successful identification of genetic loci from prostate cancer families has succeeded for those families with predisposition to early onset (<60 years of age) prostate cancer.

When the gatekeeper gene also is involved in hereditary predisposition to a cancer, the locus that cosegregates with cancer risk coincides with a chromosomal region that is often disrupted in sporadic cancer cases. This was true of both colon cancer and renal cell cancer. However, studies of genetic loci that cosegregate with prostate cancer in high-risk families have not implicated the same chromosomal loci that were disrupted in prostate cancer cells. For example, the common sites of chromosomal loss in prostate cancer cells are 7q, 8p, 10q, 16q 17p, and 22p [13-19] except for the single region at 17p, these loci do not overlap with loci for familial prostate cancer that map to chromosomes 1q24-25 [20], 1q44.2-43, [21] and Xq27-28 [22]. A hereditary prostate cancer locus has been identified at 17p11[23]. Despite the identification of several loci and, in some cases, the candidate cancer genes that reside at those loci, the mechanism by which the respective gene products influence prostate cancer is unclear. The diverse set of familial prostate cancer genes probably reflects the complex nature of prostate carcinogenesis and the diverse biology of the disease.

## **1. Genetic Loci Implicated in Studies of Prostate Cancer Families**

### **1.1 *HPC1***

*HPC1* is located on 1q24-25 and has been found to segregate with prostate cancer risk in some families with high frequency of the disease [20,24-31]. *HPC1* has been linked most definitively to families with early onset of prostate cancer, that is prior to age 60 [30,32]. A gene coding for RNaseL has been mapped to the *HPC1* locus and shown to harbor somatic mutations



in some prostate cancer cells [33]. RNaseL is involved in antiviral and proapoptotic effects induced by interferon. However, truncating mutations in the gene that codes for RNaseL are also found in controls at a lower frequency than in prostate cancer patients. This suggests that RNaseL truncation may modify risk for developing prostate cancer whether or not it is encoded by the *HPC1* gene [34]. Interestingly, gene expression profiling has implicated down regulation of the interferon pathway in prostate cancer progression providing further evidence of a possible link between a component of interferon signaling and prostate cancer pathogenesis [35]. However, thus far prostatic abnormalities have not been described in *RNaseL*-deficient mice even though those animals have an apoptosis defect in some cell lineages [36].

### 1.2 *PCAP*

A locus termed *PCAP* was found on 1q42.2-43 in one study, particularly among families with early onset prostate cancer [27,37]. Studies of other high-risk families were unable to confirm this association, although that does not exclude 1q42.2-43 as a prostate cancer locus, since alleles that are important for hereditary prostate cancer but affect a limited number of kindreds can be detected in some studies, but not others [38]. The gene encoding prostate cancer tumor antigen-1 is located on 1q42.2-43, but was not mutated in *PCAP* kindreds and therefore is probably not the *PCAP* target [39].

### 1.3 *HPCX*

A third prostate cancer susceptibility locus has been mapped to the X chromosome [22,40]. This locus has been narrowed to less than 10Mb on Xq27-28 [41]. There appears to be sufficiently strong data to implicate this locus as well as the two loci on chromosome 1 in selected prostate cancer families. However, in Iceland, that has a relatively isolated population with limited access to US gene pools, neither *HPCX* nor *HPC1* were found to be associated with

familial prostate cancer, suggesting that mutations in other genes can affect susceptibility to this tumor and that the etiology of prostate cancer is likely to be diverse with multiple genetic lesions that can predispose to neoplasia [31].

#### *1.4 ELAC/HPC2*

A fourth susceptibility locus at 17p11 was implicated when a truncating mutation was found in the gene *ELAC/HPC2* in family at high risk for prostate cancer [23]. One other family was subsequently identified with a premature stop codon in the *HPC2* gene [42]. Several missense mutations have been identified in the *HPC2* gene in the general population. These mutations influence prostate cancer risk to varying degrees, but none do so to a major degree. Risk ratios for prostate cancer conferred by the presence of these *ELAC* missense mutations are generally less than two [21,42-45]. One missense mutation, E622V, had a 1.0% prevalence in the population, but a 3.0% occurrence in men with prostate cancer (odds ratio, 2.94; 95% confidence interval, 1.05-8.23) [44]. Gene sequencing has suggested that *ELAC/HPC2* codes for a metal-dependent hydrolase that homology may be related to DNA repair enzymes [23]. Its influence in prostate cancer risk appears to be limited and may or may not be the determining risk factor in the few kindreds with mutations that result in premature chain terminations.

## **2. Oncogenes Implicated by Cytogenetic and Molecular Analyses**

### ***2.1 Haploinsufficiency of NKX3.1, a Suppressor Gene on 8p21***

Numerous oncogenes have been identified by their location of chromosomal regions commonly disrupted in specific tumors. Chromosomal locus 8p21 is the region most prone to undergo loss of heterozygosity in prostate cancer [46-53]. Furthermore, 8p21 undergoes loss in preinvasive lesions, termed PIN, as well as invasive cancer, suggesting that genes located in 8p21 are involved in cancer initiation [54]. The most thorough analysis utilizing microdissected

tissue has shown that 8p21.2 undergoes loss of heterozygosity in 85% of prostate cancer cases [55]. This makes 8p21.2 the most likely candidate for the prostate cancer gatekeeper locus. Sequence analysis of the minimal region deleted in prostate cancer, corresponding to 1.5Mb has revealed the identity of approximately 35 genes in this DNA segment [56]. Among these 10 genes that have been analyzed thus far: TRAIL receptors DR5, DR4, DcR1, DcR2, LIM-domain protein, SCAM-1 (vinexin), EGR3, LOXL2, NKX3.1, hypothetical protein KIAA0717, have not been found to harbor somatic mutations in prostate cancer specimens (C. Vocke, personal communication). Thus, the identity of the gatekeeper, as defined by the classic two-hit model, remains unconfirmed by this approach.

A leading candidate for the prostate cancer gatekeeper, the prostate-specific homeobox gene *NKX3.1*, is located nearly in the center of the minimally deleted region of 8p21.2 [56,57]. As long as five years ago *NKX3.1* was considered to be a candidate prostate tumor suppressor gene by virtue of its chromosomal location determined by FISH mapping to 8p21 [57]. However, no tumor-specific somatic mutations were found by our group or others, casting doubt on the relevance of *NKX3.1* to prostate carcinogenesis [57,58]. Nonetheless, studies with *Nkx3.1* mutant mice showed that *Nkx3.1* haploinsufficiency was semidominant, meaning that loss of a single allele conferred hyperplasia and dedifferentiation to prostate epithelial cells [59]. This raised the possibility that loss of 8p21 heterozygosity, including the *NKX3.1* gene, alone could be sufficient to disrupt the normal growth of prostate epithelial cells.

Studies of *NKX3.1* expression in human prostate cancer confirmed that the protein was expressed predominantly in the prostate gland [60,61]. However, 15% of early stage prostate cancers have complete loss of *NKX3.1* expression and expression decreases with tumor progression. Moreover, up to 80% of metastatic prostate cancer foci have lost *NKX3.1*

expression [61]. Although protein expression is lost, *NKX3.1* mRNA continues to be expressed [58,62]. This likely reflects the silencing of the remaining allele, whose coding sequence is not disrupted [57].

#### *2.1.1 Studies of Nkx3.1 in Genetically Engineered Mice*

The *NKX3.1* gene is well conserved in mammalian evolution since the murine *Nkx3.1* protein gene shares 63% amino acid identity with the human protein. *Nkx3.1* has distinct roles in murine development. Early during embryonic development *Nkx3.1* is expressed sequentially in each newly forming somite, suggesting a role in somitogenesis [63,64]. Since *Nkx3.1* deletion does not have a disabling effect on murine development, redundancies with other NK family homeoproteins probably compensate for loss of *Nkx3.1*. Near the time of birth, *Nkx3.1* expression is restricted to areas of urogenital sinus and eventually to prostatic epithelium [59,63]. Thereafter, expression of the gene is restricted to prostate and bulbourethral gland. In fact, insertion of a *lacZ* gene downstream from the *Nkx3.1* locus confers blue Xgal staining only on prostatic epithelium and the bulbourethral gland (M. Shen, personal communication). It is noteworthy that  $\beta$ -galactosidase expression was also seen in rare murine ureteral cells, thus recapitulating published findings with human tissues and suggesting that these cells may represent urothelial embryonal rests [61]

In adult mice expression of *Nkx3.1* is restricted to prostate and bulbourethral gland [59]. Moreover, murine *Nkx3.1*, like human *NKX3.1*, expression is androgen-dependent and is decreased in response to castration [63]. Studies with *Nkx3.1* gene targeted mice showed that *Nkx3.1*<sup>-/-</sup> mice were viable and fertile. However, loss of a functional murine *Nkx3.1* gene resulted in defective prostatic differentiation [59,65]. Moreover, *Nkx3.1*<sup>-/-</sup> mice developed prostatic epithelial cell hyperplasia and dysplasia later in life. *Nkx3.1* heterozygosity resulted in a similar

phenotype seen in *Nkx3.<sup>-/-</sup>* mice, but with a longer latency compared to the homozygous mutants [59].

Quite noteworthy is that conditional loss of *Nkx3.1* in mice with targeted disruption in the prostate develop lesions that resemble prostatic intraepithelial neoplasia [66,67]. Although disruption of *Nkx3.1* alone does not result in the development of prostate cancer in the mouse model, loss of *Nkx3.1* expression can cooperate with *Pten* heterozygosity to cause high frequency of prostate cancer [68]. Prostate cancers that form in *Pten<sup>+/-</sup>, Nkx3.1<sup>+/-</sup>* compound mutant mice retain the *Nkx3.1* gene, but lose Nkx3.1 protein expression further underscoring the need for loss of Nkx3.1 expression in murine prostate carcinogenesis. Moreover, the retention of *Nkx3.1* and *Nkx3.1* mRNA expression in murine prostate tumors and loss of Nkx3.1 protein expression closely recapitulate the process of gene inactivation seen in human prostate cancer. Taken together, high frequency of *NKX3.1* loss in human tumors, loss of expression with tumor progression, and mouse models demonstrating dominant *Nkx3.1* haploinsufficiency and synergy with *Pten* haploinsufficiency strongly implicate a gatekeeper role for *NKX3.1* in human prostate cancer.

### 2.1.2 Function of *NKX3.1*

NKX3.1 is a transcription factor that functions as both an activator and a repressor of target genes. For example, analysis of prostate epithelial protein expression in *Nkx3.1* mutant mice and in cultured cells showed both upward and downward alterations of gene expression [[59] and our unpublished observations]. Repression requires binding of NKX3.1 to high-affinity DNA sequences in promoter regions [69], whereas activation is achieved by DNA-independent binding of NKX3.1 to other transcription factors such as serum response factor [70]. Moreover, *NKX3.1* mutants that lack DNA binding are equally effective at transcriptional coactivation as

wild type NKX3.1 protein (our unpublished data). This implies that repression and activation functions of NKX3.1 are separable. It also implies that structural analysis of the DNA-binding motif of homeoproteins such as the parent NK-2 [71] have not provided complete information for elucidation of structure/activity relationships of the molecule that can be used to generate molecules for pharmaceutical intervention.

Human NKX3.1 is a 234 amino acid homeobox protein with the DNA-binding homeodomain in the C-terminal region of the molecule. The 60-amino acid homeodomain extends from amino acids 124-183. NKX3.1 belongs to the NK family of homeoproteins by virtue of a tyrosine (Y177) at position 54 of the 60-amino acid homeodomain [72]. This tyrosine is located in the third helix of the homeodomain. Helices 1 and 2 are aligned in parallel in the solution structure of NK family and other homeodomains [71]. The second and third helices form a helix-loop-helix structure that aligns the third helix approximately perpendicular to the first two, allowing the third helix to fit into the major groove of DNA. The third helix is responsible for DNA binding via hydrogen bonding between three amino acid regions and a hexanucleotide DNA sequence. Mutational analysis of NKX3.1 homeodomain has not been published. However, mutational analysis of NK family members has identified the amino acids KI at homeodomain positions 46 and 47, QN at homeodomain positions 50 and 51, and Y at 54 as the critical contact points between protein and DNA [72].

Control of DNA binding is not restricted to the amino acids that directly contact the major groove. For example homeodomain position 56, has been implicated in control of the length of the third helix and therefore of DNA binding [73]. Moreover, NKX3.1 binding to its cognate DNA recognition sequence is under control of phosphorylation at serine 48, a consensus protein kinase C and casein kinase II phosphorylation site [74]. Interestingly, not only is the



homeodomain the site of DNA binding, but other NK family homeoproteins have been shown to bind other transcription factors at the homeodomain as well. For example, NKX2.5 has been shown to bind SRF and the zinc finger-containing transcription factor GATA4 [75,76].

Using affinity purification chromatography we identified the hexanucleotide high-affinity DNA binding sequence for NKX3.1 – TAAGTA [69]. When these sequences were placed upstream from a minimal HSV TK promoter in a luciferase reporter construct we found that expression of NKX3.1 resulted in suppression of reporter activity [69]. In fact we have routinely found that insertion of the high-affinity DNA binding site for NKX3.1 upstream from promoters results in NKX3.1-dependent suppression of transcription. For example, when we investigated the effect of NKX3.1 on androgen-driven transcription using the MMTV LTR steroid-response element in a reporter construct, we found that the MMTV-LTR, which contains one consensus and several near consensus NKX3.1 binding sites, was suppressed by NKX3.1 (our unpublished observations). These findings are in contrast to the activity of NKX2.5, which stimulates a minimal promoter placed downstream from a triplet consensus NKX2.5 DNA binding sequence [75].

Because of some similarities in the expression of NKX3.1 and NKX2.5 during early stages of murine development, Carson et al examined the effect of NKX3.1 on the activity of the smooth muscle  $\gamma$ -actin (SMGA) promoter [70]. SMGA is expressed during somitogenesis and has been shown to be controlled by NKX2.5 which acts as a transcriptional coactivator for SMGA by binding to SRF [75]. Binding of NKX2.5 to SRF is DNA-independent. In this manner, NKX2.5 acts as a transcriptional coactivator for SRF and will augment the effect of SRF on promoters that contain serum response element (SRE) motifs, the DNA binding regions for SRF. Similarly, Carson found that NKX3.1 bound to SRF and enhanced activation of the SMGA

promoter in reporter gene assays [70]. NKX3.1 can also inhibit the activity of transcription factors. Recently another transcription factor, the prostate-derived ETS-factor (PEDF), was isolated by a yeast two-hybrid screening using Nkx3.1 as bait. In reporter gene assays Nkx3.1 was found to repress transcriptional activation by PDEF [77]. Taken together, the data on transcriptional activation by NKX3.1 suggest that when NKX3.1 binds to high-affinity DNA binding sites it inhibits transcription. But NKX3.1 can also bind to serve as a coactivator for other transcription factors and thereby can activate or repress gene expression. Much additional work will be needed to determine the spectrum of transcription factors that interact with NKX3.1.

## *2.2 Altered Expression of PTEN, a Suppressor Gene on 10q24*

### *2.2.1 PTEN in Human Prostate Cancer*

Loss of heterozygosity and karyotypic abnormalities at 10q24 have been well demonstrated in a substantial fraction of prostate cancers [15,16,19,78]. This region was quite similar to the 10q24 deletions seen in gliomas and therefore when an oncogene, *PTEN*, that mapped to 10q24 was found in gliomas, it was immediately a candidate tumor suppressor for prostate cancer and other cancers [79,80,80-82]. *PTEN* codes for a lipid phosphatase that cleaves 3-phosphorylated phosphoinositides and thereby functions as a negative regulator of phosphoinositol-3-kinase signaling [83-86]. The lipid phosphatase activity of PTEN protein is essential for its tumor suppressor functions [87,88]. Downstream from PTEN is the kinase AKT1/PKB that controls apoptosis by phosphorylation of the proapoptotic protein BAD [89]. AKT1/PKB also exerts controls over cell cycle progression by phosphorylation of P27 [90-92]. Therefore PTEN has the capacity to influence both cell death and cell growth signaling in cells.

*PTEN* undergoes loss of heterozygosity in a substantial fraction of prostate cancer specimens [93,94]. Tumor progression to advanced prostate cancer may be accompanied by reduced PTEN protein expression caused by a variety of mechanisms that may or may not result from gene inactivation [95,96]. Consistent with its role in advanced prostate cancer, *PTEN* gene alterations occur sporadically in different foci of metastatic prostate cancer indicating that its inactivation is probably not the gatekeeper event that initiate prostatic neoplasia [97]. Primary prostate cancers display a low frequency of *PTEN* gene inactivation [98-100] and analyses of prostate cancers from Asian countries has also shown a low level of *PTEN* mutations in specimens from early-stage patients [101,102]. Targeted deletion of *Pten* in the mouse results in embryonic lethality when both alleles are deleted and causes susceptibility to a variety of tumors, particularly lymphoid malignancies [103]. *Pten* mutant mice develop prostate neoplasms [104,105] and *Pten* heterozygosity accelerates the development of prostate cancers in the TRAMP transgenic mouse model of prostate cancer [106].

### 2.2.2 Prostate Neoplasia in *Pten* Gene-Targeted Mice

Importantly, *Pten* heterozygosity can cooperate with other oncogenes that have been implicated in human prostate cancer. *Pten*<sup>+/-</sup>/*Cdkn1b*<sup>-/-</sup> mutant mice with no expression of the *Cdkn1b* gene product, p27/Kip1, develop prostate cancers early in life that simulate the progression of prostate neoplasia seen in human cancer [107]. *Pten* heterozygosity also cooperates with *Nkx3.1* heterozygosity as described above [66,68]. The mouse model confirms that *Pten* is important for prostate cancer development and is likely to play a role in human prostate cancer progression. Loss of *Pten* also interacts with *Nkx3.1* heterozygosity to enhance prostate tumorigenesis as described above. Loss of *Pten* expression may potentiate tumor angiogenesis [108,109]. Moreover, PTEN plays a role in modifying androgen receptor signaling

which is perhaps the most influential and universally preserved growth pathway for advanced prostate carcinoma [110,111]. *PTEN* plays an infrequent role in early human prostate cancer and no *PTEN* mutations have been identified in prostate cancer families [112,113].

### **3. *PRC17* A GAP-Family Protein on 17q12**

Studies of genomic amplification in prostate cancer identified a locus on 17q12 that is amplified in about 15% of prostate cancers. The gene *PRC17* is found at that locus and is over expressed in about one half of metastatic prostate cancers [114]. The gene codes for a protein that belongs to the family of GTPase activating proteins. The GTPase activation activity is responsible for its transforming activity. *PRC17* is a likely candidate prostate cancer oncogene in advanced disease and perhaps for some early stage cancers.

### **4. Genes Implicated in Experimental Models**

#### **4.1 *EGR1***

The early growth response gene *EGR1* codes for is a transcription factor in the family of WT1 proteins. The Zn-finger domain of WT1 binds to a GC-rich *EGR1*-like DNA-binding element [115]. The GC-rich *EGR1*-binding element regulates the E-cadherin promoter, among other genes, an association that may be relevant in prostate cancer [116]. Expression of the androgen receptor gene is inhibited by binding of WT1 to either GC-rich *EGR1*-like sequences or TCC repeats located in the promoter region of the *AR* gene [117].

Of great interest is the fact that the early growth response gene product, EGR-1, is a transcription factor that binds to DNA sequences similar to those that bind WT-1 [118,119]. *EGR1* is over expressed in some prostate cancers and controls the expression of genes involved in cell growth and neural differentiation when transfected into prostate cells [120]. *EGR1* can also sustain cell growth and activate expression of proangiogenic proteins [121]. *Egr-1* gene

deletion in mice results in suppression of prostate tumorigenesis caused by the SV40 T-antigen transgene of the TRAMP mouse [122]. EGR1 function has been implicated in human prostate cancer because expression of NAB2, an EGR1 corepressor, is lost in many prostate cancer tissues [123].

#### 4.2 *RASSF1A*

The growth and antiapoptotic pathways mediated by one or more members of the RAS family of oncoproteins are commonly activated in human cancers. However, mutations in the *K-RAS* gene are often found in cancers associated with carcinogenic insults such as smoking, but are not commonly found in prostate cancer [124]. The RASSF1 proteins have been shown to inhibit RAS activity and provide a proapoptotic signal to cells [125]. *RASSF1* maps to a locus on chromosome 3p21.3 gene and its expression is down regulated by gene methylation in prostate cancer [126,127]. The signals that activate methylation of the *RASSF1A* promoter and inactivate the 3p21.3 locus in prostate cancer remains to be elucidated.

### 5. Conclusions

A variety of approaches have identified several candidate loci and genes that have been implicated in prostate carcinogenesis. These are summarized in Table 1. Much additional work will be required to understand the predominant genetic programs underlying prostate cancer. Regarding genetic loci that segregate with familial prostate cancer risk, not only do the target genes have to be identified definitively, but their mechanisms of action have to be determined. The use of gene targeting studies in mice will also help to confirm the pathogenic role that some of these genes have in prostate carcinogenesis. Obviously substantial work needs to be done to sort out the genetic programs that lead to prostate cancer.

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**Table 1**  
**Summary of Genetic Loci Implicated in Prostate Cancer Pathogenesis**

<b>Gene</b>	<b>Alternative Name</b>	<b>Locus</b>	<b>Cancer Association<sup>a</sup></b>	<b>Genetic Alteration in Cancer</b>	<b>Altered Expression in Cancer<sup>b</sup></b>
<i>HPC1</i>	RNaseL	1q24-25	Familial	Mutated	Rare
<i>PCAP</i>		1q42.2-43	Familial	ND <sup>c</sup>	ND
<i>HPCX</i>		Xq27-28	Familial	ND	ND
<i>HPC2</i>	<i>ELAC</i>	17p11	Familial	ND	ND
<i>PTEN</i>		10q24	Sporadic	Deleted, Mutated	Yes
<i>NKX3.1</i>		8p21.2	Sporadic	Deleted	Yes
<i>PRC17</i>		17q12	Sporadic	Amplified	Yes

<sup>a</sup> Indicates whether gene was found by linkage analysis or genetic analysis of cancer cells.

<sup>b</sup> Altered expression determined by either RT-PCR or by measurement of protein.

<sup>c</sup> ND – not determined.